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Antidepressant-like effect of icariin and its possible mechanism in mice

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Abstract

The behavioral, neurochemical and neuroendocrine effects of icariin isolated from *Epimedium brevicornum* were investigated in behavioral despair models of KunMing strain of male mice. Icariin was found to significantly shorten immobility time in the forced swimming test (FST) after orally administration for 21 consecutive days. Icarrin also produced a marked reduction in immobility time in the tail suspension test (TST) when administered for at least 7 consecutive days. The preferable antidepressant action by icariin was obtained at 17.5 and 35 mg/kg in the present study. Moreover, it was observed that the stress of FST exposure induced increases in brain monoamine oxidase (MAO) A and B activities, serum corticotropin-releasing factor (CRF) levels, as well as decreases in brain monoamine neurotransmitter levels. Treatment of icariin for 21 consecutive days mainly reversed the above effects in the mouse FST. These results suggested that icarrin possessed potent antidepressant-like properties that were mediated via neurochemical and neuroendocrine systems.

Keywords: Icariin; Forced swimming test; Tail suspension test; Monoamine neurotransmitters; Monoamine oxidase; Corticotropin-releasing factor

1. Introduction

Depressive disorders are common and often disabling. Monoamine neurotransmitters including serotonin (5-HT), noradrenaline (NA) and dopamine (DA) are believed to be involved in mental depression and play important roles in mediating behavioral effects of antidepressant drugs. Monoamine oxidase (MAO) is a key enzyme that is associated with the metabolism of these neurotransmitters. Its activity has been suggested to be a trait-dependent indicator of vulnerability to psychopathology (Haier et al., 1988). The function of the hypothalamic-pituitary-adrenal (HPA) axis was impaired in many depressed patients (Barden et al., 1995). Corticotropin-releasing factor (CRF), as the major physiological regulator of the HPA axis system, acts within the central nervous system to modulate a number of behavioral, neuroendocrine and autonomic responses to environmental stimulation (Arborelius et al., 1999). In addition, the hyperactivity of CRF system appears to be a state marker for depression because the overactivity of the HPA axis normalizes following successful antidepressant treatment (Arborelius et al., 1999). There was a strong relationship between MAO activity and the HPA axis function in depressed patients (Pandey et al., 1992). Furthermore, these patients presented both a reduced monoaminergic activity and a hyperactivity of the HPA axis. Treatments with certain selective serotonin reuptake inhibitors (SSRIs) have been shown to reduce the activity of CRF neurons and may contribute to their therapeutic action (Nemeroff and Owens, 2004). In animal models of depression, it was confirmed that endogenous CRF modulated 5-HT transmission (Price et al., 2002), meanwhile, SSRI fluoxetine and tricyclic antidepressant amitriptyline decreased hypothalamic CRF mRNA levels (Aubry et al., 1999; Damjanoska et al., 2003). These findings strongly supported that there were functional interactions between monoamergic system and the HPA axis system in brain (Linthorst et al., 2002; Price et al., 2002).

Icariin (Fig. 1) is a major constituent of flavonoids isolated from *Epimedium brevicornum* Maxim (Berberidaceae), which is used as a traditional Chinese medicine to nourish the *kidney* and reinforce *yang*. Icariin and *E. brevicornum* have a wide range of pharmacological and biological activities, including

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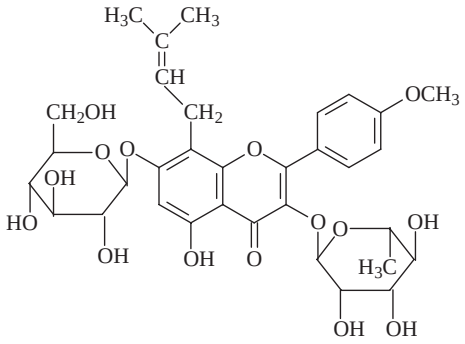

Fig. 1. Structure of icariin.

regulating cardiovascular, circulatory, genital, and bone marrow systems, stimulating neurite growth and possessing estrogenic activity (Kuroda et al., 2000; Wu et al., 2003; Wang and Lou, 2004; Liu et al., 2005; Ye et al., 2005). It was observed that total flavonoids extracted from *E. brevicornum* reversed the attenuations of monoamine neurotransmitters such as 5-HT, 5-Hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT, NA and DA, and regulated the gene expression of neruotransmitter receptors in hypothalamus of the old rats (Meng and Zeng, 1996; Shen et al., 2004). Clinical evident suggested that *E. brevicornum* could improve depressive symptoms after stroke (Lai, 2001). According to all the above observations, we hypothesized that icariin may act by inhibiting MAO activity, mediating monoamine neurotransmitter levels and regulating CRF system in response to stress and depression.

The aim of the present study was to determine whether icariin showed antidepressant activity in behavioral despair models of animals. The mouse forced swimming test (FST) and tail suspension test (TST) are widely used to predict antidepressant efficacy indicated by immobility time to be reduced by several different classes of antidepressant drugs (Porsolt et al., 1977; Steru et al., 1985). Therefore, these depression models were used in the present study to assess the potential efficacy of icarrin. In order to investigate possible time-dependent effects on immobility time, oral treatments with icariin for 1, 3, 7, 14 and 21 consecutive days were investigated under the standardized application schedule, preceded by the appropriate vehicle control application, respectively. It is well known that the FST can be used to explore concomitant changes in neurochemical and neuroendocrine systems (Miura et al., 1996; Connor et al., 1997; Linthorst et al., 2002; Drossopoulou et al., 2004; Chen et al., 2005; Petit-Demouliere et al., 2005). To substantiate the hypothesis that any antidepressant-like activity obtained with icariin resulted from the regulation of neurochemical and neuroendocrine systems, the effects of oral 21-consecutive-day treatment with icariin on brain MAO activity was studied in the mouse FST model. This was also accomplished by assessing its effects on whole brain 5-HT, 5-HIAA, NA and DA levels. Moreover, it was of interest to determine whether icariin display normalization of the HPA axis activity in the FST model via reducing serum CRF levels.

2. Materials and methods

2.1. Reagents

Icariin was purchased from Bio-sep Bio-technique Stock Co., Ltd. Xi'an Jiaotong University (P.R. China). The purity of icariin was checked by high-performance liquid chromatography (HPLC) to be at least 98% pure (Wang et al., 2003). Fluoxetine hydrochloride and amitriptyline hydrochloride were from Changzhou Siyao Pharmaceuticals Co., Ltd. (P.R. China). 5-HT, 5-HIAA, NA, DA and β-phenylethylamine (PEA) were purchased from Sigma (St. Louis, MO, USA). Other reagents were analytical grades made in P.R. China.

2.2. Animals

Male KunMing strain of mice (Laboratory Animal Centre, Jiangsu Province, P.R. China), weighing 23–25 g, were used in this study. Animals were housed 8 per cage ($320 \times 180 \times 160$ cm) under a normal 12 h:12 h light/dark schedule with the lights on at 07:00 a.m. and had free access to tap water and food pellets. Ambient temperature and relative humidity were maintained at 22 ± 2 °C and at $55 \pm 5\%$, respectively. They were allowed at least one week to adapt to the laboratory environment before experiments. Experiments were carried out between 1:00 p.m. and 4:00 p.m. in a room adjacent to that in which the mice were housed under the same conditions of temperature, humidity, and light cycle, and to which the animals had been brought at least 30 min prior to the experiment to avoid alterations in their behaviours due to their transfer from one room to another. All studies were carried out in accordance with the Institutional Animal Care Committee at the Nanjing University and the China council on Animal Care at Nanjing University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Drug administration

Different groups of mice were used for drug treatment and for each test. Drugs were dissolved or dispersed in saline, respectively. All doses were expressed as milligrams per kilogram body weight of the respective drugs. Food, but not water, was withdrawn from the animals 1 h prior to drug administration.

Icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg), amitriptyline (10 mg/kg) and saline (15 ml/kg) were administered by gastric gavage once daily at 11:00 a.m. to 12:00 p.m. for 1, 3, 7, 14 and 21 consecutive days to different groups of animals (12 animals per group) in the FST and TST, respectively. The behavioral tests were conducted 1 h after the last treatment of drugs. To study the effects of swim stress on biochemical index, saline (normal control and FST control), icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) were orally administered once daily for 21 consecutive days to mice (12 animals per group), respectively, then mice were subjected a FST 1 h after the last

treatment. To study the effects of these tested drugs on biochemical index in unstressed animals, saline, icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) were orally treated once daily for 21 consecutive days to animals (8 animals per group), respectively.

2.4. Forced swimming test (FST) in mice

The FST used was the same as described in detail elsewhere (Porsolt et al., 1977). Briefly, mice were individually placed in a glass cylinder (20 cm in height, 14 cm in diameter) filled with 10-cm high of water (25±2 °C). Each mouse was given a 6-min swimming test, and the duration of immobility was observed and measured during the final 4-min interval of the test. All test swim sessions were recorded by a video camera positioned directly above the cylinder. Two competent observers, who were unaware of the treatment each mouse had received, scored the videotapes. Immobility period was regarded as the time spent by the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water. Following swimming sessions, they were then towel dried and returned to their housing condition. The animals were used only once in this test.

2.5. Tail suspension test (TST) in mice

The TST was conducted as previously described (Steru et al., 1985). Briefly, mice were individually suspended by tail with clamp (1 cm from the tip of the end) in a box (25 × 25 × 30 cm) with the head 5 cm to the bottom. Testing was carried out in a darkened room with minimal background noise. All animals were suspended for total 6 min, and the duration of immobility was observed and measured during the final 4-min interval of the test. All test sessions were recorded by a video camera positioned directly above the box. Two competent observers blind to treatment scored the videotapes. Mice consider immobile only when they hung passively and completely motionless. The animals were used only once in this test.

2.6. Blood and brain collection

Eight groups of mice given saline (normal control and FST control), icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) once daily for 21 days, respectively, were sacrificed immediately after exposure to the FST. In addition, seven groups of the unstressed animals given saline, icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) once daily for 21 days, were also sacrificed immediately, respectively. The blood was collected on ice and separated in a refrigerated centrifuge at 4 °C. Serum was stored at −80 °C until assays were performed. Following blood collection, their right and left dissected brains were rapidly removed and immediately frozen on dry ice and stored at −80 °C until biochemical assays.

2.7. Biochemical measurements

2.7.1. MAO-A and MAO-B activities

Mouse brain mitochondrial fractions were prepared following the procedure described previously (Schurr and Livne, 1976). MAO activity was assessed spectrophotometrically as described previously (Yu et al., 2002). Briefly, the mitochondrial fraction suspended in 9 vol. of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose), was mingled at 4 °C for 20 min. The mixture was centrifuged at 4000 rpm for 10 min at 4 °C and the supernatant was re-centrifuged to get the protein deposition. Then, the deposition was re-suspended in the same buffer. The protein concentration was adjusted to 1 mg/ml. Protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. The assay mixtures contained 4 mM 5-HT or 2 mM β-PEA as specific substrates for MAO-A and MAO-B, respectively, 200 μl solution of the mitochondrial fraction, and 10 mM sodium phosphate buffer (pH 7.4) up to a final volume of 1 ml. The reaction was allowed to proceed at 37 °C for 20 min, and stopped by adding 200 μl of 1M HCl, the reaction product was extracted with 4 ml of butylacetate (for MAO-A assay) or cyclohexane (for MAO-B assay), respectively. The organic phase was measured at wavelength of 280 nm and 242 nm for MAO-A and MAO-B assay with spectrophotometer, respectively. Blank samples were prepared by adding 200 μl of 1 M HCl prior to reaction, and worked up subsequently in the same manner.

2.7.2. Monoamine neurotransmitter levels

Monoamine neurotransmitters 5-HT, 5-HIAA, NA and DA contents in mouse whole brain were measured by HPLC coupled with electrochemical detection (Alburges et al., 1993). The brain tissues (150–250 mg) were homogenized in an ice-cold solution of 0.4 M perchloric acid (6.8 μl/mg) containing 5 mM sodium bisulfite and 0.04 mM EDTA for avoiding oxidation, using a Polytron homogenizer, and then centrifuged at 30,000×*g* for 15 min at 4 °C. 10 μl of the resulting supernatant was chromatographed on a Luna C18(2) RP-18 column (150 × 4.6 mm; 5 μm) protected by C_{18} Phenomenex precolumn (Phenomenex, USA) in a Waters 600 liquid chromatography. The separation was done in an isocratic elution mode at column temperature 20 °C using a mobile phase consisting of 17.6% methanol (v/v) and 82.4% distilled water containing 0.0876 mM EDTA2Na, 1.512 mM triethylamine, 9 mM DL-10-camphorsulfonic acid, 20 mM $Na_2HPO_4{\cdot}12H_2O$ and 15 mM citrate, at a flow rate of 0.7 ml/min. The measurements were done at electrode potentials of a glassy carbon electrode +650 mV vs. Ag/AgCl reference electrode with Waters 464 electrochemical detector. 5-HT, 5-HIAA, NA and DA were identified and quantified by comparing their retention times and peak areas to those of standard solutions. The concentrations of 5-HT, 5-HIAA, NA and DA were expressed in ng/g wet weight tissue.

Identification (by retention time) and measurement of the compounds (by peak area) in the samples was achieved by comparison to 0.2–4 ng/ ml 5-HT, 5-HIAA, NA and DA

standard solutions. 5-HT, 5-HIAA, NA and DA were prepared as 0.5 mg/ml stock solution in 0.4 M perchloric acid.

2.7.3. CRF levels

Serum CRF levels were determined by using a commercially available radio-immunoassay kits (Technique center of radioimmunity of Navy in Beijing P.R. China, Beijing) in Immunoassay department of Inspection department, Nanjing General Hospital of Nanjing Military Command, P.R. China. The sensitivity of the assay was 0.2 ng/ml. Intra- and inter-assay variabilities for these assays were less than 8%.

2.8. Statistical analyses

Results were presented as the mean±SEM. The behavioral data were analyzed by using one-way ANOVA followed by Least-significant difference (LSD) Student–Newman–Keuls's or Dunnett's T3 post hoc test, for individual between-group comparisons. Treatment effects on biochemical parameters were assessed with one-way or two-way ANOVA. A probability level of 0.05 or less was accepted as significant.

3. Results

3.1. Effects of icariin on immobility time in the mouse FST

The effects of icariin, fluoxetine and amitriptyline on immobility time in the mouse FST were shown in Fig. 2. There was no significant change in immobility time after 1-, 3-, 7-day treatment with icariin dosing from 8.75 to 70 mg/kg [1-day: $F(4, 55)=0.488$, $P=0.744$; 3-day: $F(4, 55)=0.513$, $P=0.727$; 7-day: $F(4, 55)=0.680$, $P=0.609$]. Only icarrin at 17.5 mg/kg exhibited a significant decrease in immobility

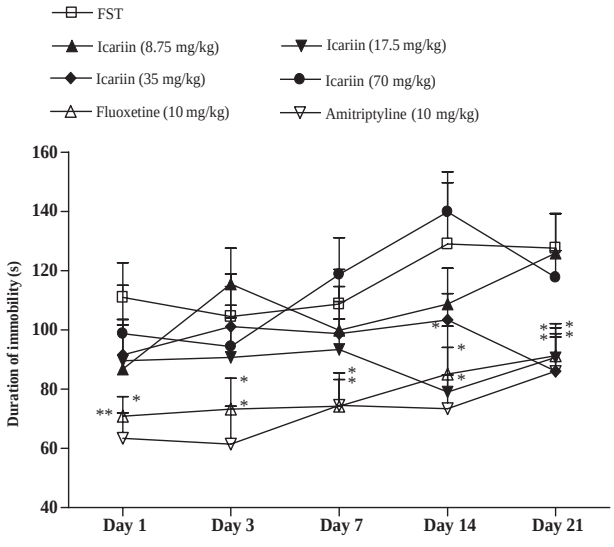

Fig. 2. Effects of icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) on immobility time in the mouse FST. They were orally administrated for 1, 3, 7, 14 and 21 consecutive days, respectively. The data were given as the mean±SEM. *$P<0.05$, **$P<0.01$ when compared to the control animals (vehicle+saline). The number of animals in each group was 12.

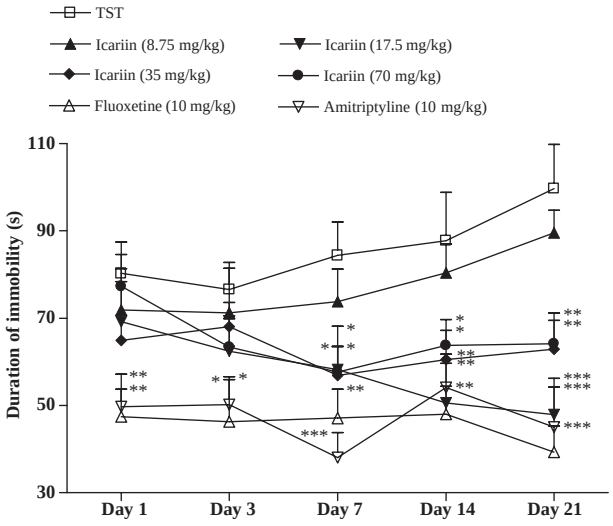

Fig. 3. Effects of icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) on immobility time in the mouse TST. They were orally administrated for 1, 3, 7, 14 and 21 consecutive days, respectively. The data were given as the mean±SEM. *$P<0.05$, **$P<0.01$, ***P<0.001 when compared to the control animals (vehicle+saline). The number of animals in each group was 12.

duration after oral administration for 14 days [$F(1, 22)=4.401$, $P=0.047$]. After 21-day treatment of icariin, there was a significant treatment effect for dose in immobility time. The maximal effect was obtained at 35 mg/kg. As be respected, the reference antidepressant fluoxetine and amitriptyline at 10 mg/kg induced a marked reduction in immobility time at the tested time points, respectively.

3.2. Effects of icariin on immobility time in the mouse TST

The effects of icariin, fluoxetine and amitriptyline on immobility time in the mouse TST were shown in Fig. 3. There was no significant change of immobility time in the mouse TST for 1- and 3-day treatment of icariin at the doses from 8.75 to 70 mg/kg [1-day: $F(4, 55)=0.608$, $P=0.659$; 3-day: $F(4, 55)=0.608$, $P=0.659$]. Icariin produced a decrease in immobility time after 7-day treatment, the minimum effective dose (MED) value being 35 mg/kg [$F(1, 22)=7.318$, $P=0.013$]. After 14- and 21-day treatment, icariin also affected the duration, the maximal effect was obtained at 17.5 mg/kg [14-day: $F(1, 22)=6.663$, $P=0.017$; 21-day: $F(1, 22)=15.682$, $P=0.001$]. However, icariin at the lowest dose did not show any TST behavioural effect in mice. Fluoxetine and amitriptyline resulted in a significant reduction at each dosed point.

3.3. Effects of icariin on whole brain MAO-A and MAO-B activities in stressed and normal mice

The effects of icariin, fluoxetine and amitriptyline after 21 consecutive day treatments on whole brain MAO-A and MAO-B activities in stressed and normal mice were showed in Fig. 4. Significant increases in whole brain MAO-A and MAO-B activities were observed in mice exposed to swim stress procedure [MAO-A: $F(1, 22)=48.779$, $P<0.001$; MAO-B:

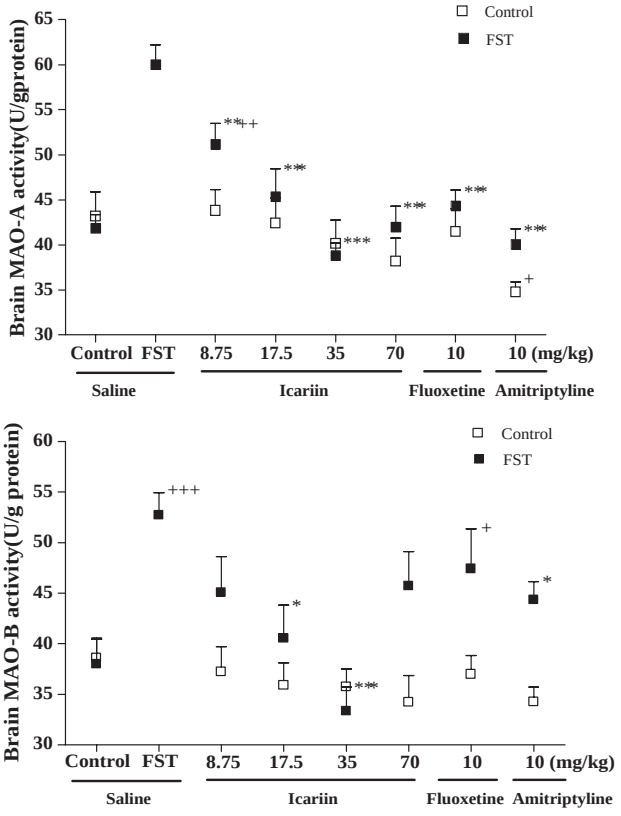

Fig. 4. Effects of icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) on brain MAO-A (Part A) and MAO-B (Part B) activities in stressed and normal mice. They were orally administrated for 21 consecutive days, respectively. The data were given as the mean±SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared to the stressed animals (vehicle+saline). $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ when compared to the normal animals (vehicle+saline), respectively. The numbers of animals in stressed and normal groups were 12 and 8, respectively.

$F(1, 22)=18.975$, $P<0.001$]. The overall effect of 21 days of icariin treatment led to significant effects of Treatment [$F(4, 90)=8.391$, $P<0.001$], Stress [$F(1, 90)=14.073$, $P<0.001$] and Treatment × Stress interaction [$F(4, 90)=3.761$, $P=0.007$] on MAO-A activity, and significant effects of Treatment [$F(4, 90)=3.872$, $P=0.006$], Stress [$F(1, 90)=14.973$, $P<0.001$], nonsignificant Treatment × Stress interaction effect [$F(4, 90)=2.425$, $P=0.054$] on MAO-B activity. Icariin at the doses from 8.75 to 70 mg/kg, markedly blocked brain MAO-A and MAO-B activities compared with the stress vehicle, respectively [8.75 mg/kg: MAO-A: $F(1, 22)=7.898$, $P=0.010$; MAO-B: $F(1, 22)=3.329$, $P=0.082$; 17.5 mg/kg: MAO-A: $F(1, 22)=15.289$, $P=0.001$; MAO-B: $F(1, 22)=9.514$, $P=0.005$; 35 mg/kg: MAO-A: $F(1, 22)=69.620$, $P<0.001$; MAO-B: $F(1, 22)=35.221$, $P<0.001$;70 mg/kg: MAO-A: $F(1, 22)=32.362$, $P<0.001$; MAO-B: $F(1, 22)=2.965$, $P=0.099$]. The maximal effect was obtained at 35 mg/kg. Moreover, icariin at the doses from 8.75 to 35 mg/kg showed similar effects on both MAO-A and MAO-B activities. However, icariin at 70 mg/kg was more potent for MAO-A activity than MAO-B activity. Only icariin at 35 mg/kg was capable of reversing MAO-A and MAO-B activities to the value below the normal. Fluoxetine at 10 mg/kg only depressed brain MAO-A activity induced by the FST [MAO-A: $F(1, 22)=32.137$, $P<0.001$; MAO-B: $F(1, 22)=1.370$, $P=0.254$]. As expected, amitriptyline at 10 mg/kg significantly blocked brain MAO-A and MAO-B activities in the mouse FST [MAO-A: $F(1, 22)=52.657$, $P<0.001$; MAO-B: $F(1, 22)=8.597$, $P=0.008$], respectively.

In unstressed animals, icarrin at 70 mg/kg trended to inhibit brain MAO-A activity [$F(1, 14)=1.834$, $P=0.197$]. Amitriptyline remarkably reduced the MAO-A activity [$F(1, 14)=8.475$, $P=0.011$].

3.4. Effects of icariin on whole brain monoamine neurotransmitter levels in stressed and normal mice

The effects of icariin, fluoxetine and amitriptyline after 21 consecutive day treatments on whole brain monoamine neurotransmitter levels in stressed and normal mice were summarized in Table 1. Significant decreases in 5-HT and 5-HIAA levels in whole mouse brain were observed after stress of FST procedure [5-HT: $F(1, 22)=15.677$, $P=0.001$; 5-HIAA: $F(1, 22)=5.042$, $P=0.035$], resulting in a marked effect on the 5-HIAA/5-HT ratio [$F(1, 22)=4.411$, $P=0.049$]. The overall effect of 21 days of icariin treatment led to significant Treatment effect on 5-HT and 5-HIAA/5-HT [$F(4, 90)=18.024$, $P<0.001$; $F(4, 90)=2.548$, $P=0.045$, respectively], and nonsignificant effects of Stress and Treatment × Stress interaction [5-HT: $F(1, 90)=0.361$, $P=0.549$; $F(4, 90)=0.773$, $P=0.546$; 5-HIAA/5-HT: $F(1, 90)=0.869$, $P=0.354$; $F(4, 90)=1.871$, $P=0.122$]. In addition, both Treatment [$F(4, 90)=2.460$, $P=0.051$] and Stress [$F(1, 90)=2.373$, $P=0.127$] had no significant effect on 5-HIAA, however, there was statistically significant Treatment × Stress interaction effect on 5-HIAA [$F(4, 90)=3.866$, $P=0.006$]. Icariin markedly elevated brain 5-HT levels [8.75 mg/kg: $F(1, 22)=47.496$, $P<0.001$; 17.5 mg/kg: $F(1, 22)=47.643$, $P<0.001$; 35 mg/kg: $F(1, 22)=7.676$, $P=0.011$; 70 mg/kg: $F(1, 22)=30.953$, $P<0.001$]. Icariin at 8.75 and 17.5 mg/kg attenuated the swim induced decrease in 5-HT level to exceed the normal value. They also significantly elevated brain 5-HIAA levels [8.75 mg/kg: $F(1, 22)=15.438$, $P=0.001$; 17.5 mg/kg: $F(1, 22)=13.806$, $P=0.002$]. The latter strongly reversed the decreased 5-HIAA levels to exceed the normal value. Icariin attenuated this swim stress-induced increase in the 5-HIAA/5-HT ratio, with MED value being 17.5 mg/kg [$F(1, 22)=4.747$, $P=0.045$]. In the cases of icariin at 17.5 mg/kg, the changes in 5-HT turnover were predominantly due to increases both in brain 5-HT and 5-HIAA levels. However, in the cases of icariin at 35 and 70 mg/kg, the alterations in 5-HT turnover were predominantly due to changes in brain 5-HT levels, without any significant variety in brain 5-HIAA concentrations. 5-HT contents in brain were significantly increased, but the 5-HIAA contents were decreased in fluoxetine-treated mice compared to stressed-animal control in this study [Compared with stress vehicle: 5-HT: $F(1, 22)=4.625$, $P=0.049$; 5-HIAA: $F(1, 22)=2.256$, $P=0.155$. Compared with normal vehicle: 5-HIAA: $F(1, 22)=15.216$, $P=0.001$]. As expected, fluoxetine produced a significant

Table 1
Effects of icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) on brain monoamine neurotransmitter levels and 5-HIAA/5-HT ratio in stressed and normal mice

Group	Dose (mg/kg)	monoamine neurotransmitter levels (ng/g wet tissue)				5-HIAA/5-HT ratio
		5-HT	5-HIAA	NA	DA	
Normal vehicle	–	913.5±52.9	299.3±14.8	401.0±23.7	1251.8±84.9	0.328±0.017
Stress Vehicle	–	620.9±39.0^{+++}	242.5±21.1^{+}	296.2±28.3^{++}	1028.6±72.0	0.391±0.024^{+}
Icariin	8.75	1018.5±42.5***	340.2±9.4**	453.9±29.5***	1167.5±78.1	0.337±0.013
	17.5	1048.5±47.1***	356.7±23.5***$^{+}$	448.5±34.6**	1264.5±73.1*	0.328±0.016*
	35	835.8±40.2*	273.0±19.0	457.3±26.5***	1384.9±98.7**	0.333±0.007
	70	990.5±55.2***	283.7±20.4	500.3±28.0***$^{+}$	1481.0±87.1***	0.304±0.021**
Fluoxetine	10	785.1±71.0*	197.9±19.8^{++}	354.8±15.4	1107.5±127.5	0.252±0.024**$^{+}$
Amitriptyline	10	756.3±97.8^{+}	290.7±30.3	374.8±39.4	1056.9±67.4	0.384±0.036^{+}
Normal vehicle	–	710.8±41.1	220.2±19.8	307.6±32.2	1097.5±75.8	0.310±0.024
Icariin	8.75	893.4±47.3	246.7±9.5	313.1±29.9	956.6±95.2	0.276±0.029
	17.5	1106.9±37.3	223.5±19.6	342.7±39.0	826.3±103.4^{+}	0.202±0.018^{+}
	35	1031.7±43.0	312.5±39.0	342.6±46.3	1060.5±95.4	0.303±0.025
	70	957.4±57.5	339.6±31.6^{+}	370.4±33.4	994.0±84.0	0.355±0.037
Fluoxetine	10	978.8±21.3	188.4±26.8	260.6±32.6	922.0±109.8	0.192±0.024^{+}
Amitriptyline	10	791.4±89.0	324.4±31.2	259.0±12.6	998.8±91.8	0.410±0.056

They were orally administrated for 21 consecutive days, respectively. The data were given as the mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared to the stressed animals (vehicle+saline). $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ when compared to the normal animals (vehicle+saline), respectively. The numbers of animals in stressed and normal groups were 12 and 8, respectively.

decrease in the 5-HIAA/5-HT ratio [$F(1, 22)=15.806$, $P=0.001$]. Amitriptyline trended to increase brain 5-HT and 5-HIAA contents in the mouse FST [5-HT: $F(1, 22)=1.806$, $P=0.199$; 5-HIAA: $F(1, 22)=1.762$, $P=0.204$]. However, it significantly elevated 5-HT turnover to exceed the normal [$F(1, 22)=4.335$, $P=0.049$].

Swim stress markedly reduced brain NA levels [$F(1, 22)=8.137$, $P=0.009$]. The overall effect of 21 days of icariin treatment led to significant effects of Stress [$F(1, 90)=463.323$, $P<0.001$] and Treatment × Stress interaction [$F(4, 90)=5.744$, $P<0.001$], and nonsignificant Treatment effect [$F(4, 90)=2.325$, $P=0.062$]. In stress animals, icariin elicited to increase the NA levels with MED value being 8.75 mg/kg [$F(1, 22)=15.295$, $P=0.001$]. In addition, icariin at the highest dose significantly increased the levels to exceed the normal [Compared with stress vehicle: $F(1, 22)=26.105$, $P< 0.001$; Compared with normal vehicle: $F(1, 22)=7.348$, $P=0.013$]. Fluoxetine and amitriptyline failed to significantly alter [$F(2, 33)=1.865$, $P=0.181$].

Although decreased brain DA concentrations in the mouse FST were not significantly different from the normal control [$F(1, 22)=3.806$, $P=0.067$], the overall effect of 21 days of icariin treatment led to significant effects of Stress [$F(1, 90)=117.431$, $P<0.001$] and Treatment × Stress interaction [$F(4, 90)=2.731$, $P=0.034$], and nonsignificant Treatment effect [$F(4, 90)=0.565$, $P=0.689$]. Icariin exhibited to elevate brain DA levels with MED value being17.5 mg/kg [$F(1, 22)=5.091$, $P=0.035$]. Icariin at 35 and 70 mg/kg increased the levels to the normal value [35 mg/kg: $F(1, 22)=7.722$, $P=0.011$; 70 mg/kg: $F(1, 22)=15.604$, $P=0.001$]. Fluoxetine and amitriptyline were without significant effect [Fluoxetine: $F(1, 22)=0.197$, $P=0.823$; Amitriptyline: $F(1, 22)=1.865$, $P=0.181$].

In unstressed animals, icariin at 17.5 and 35 mg/kg slightly but not significantly enhance brain 5-HT levels [$F(2, 21)=2.758$, $P=0.085$]. The former did not alter 5-HTAA levels [$F(1, 14)=0.400$, $P=0.675$], however, the latter exhibited a tendency to increase 5-HIAA levels [$F(1, 14)=3.070$, $P=0.099$]. Only the dose 17.5 mg/kg of icariin produced a significant reduction in the 5-HIAA/5-HT ratio [$F(1, 14)=4.475$, $P=0.041$]. Icariin at 70 mg/kg did not showed any change in the 5-HT levels [$F(1, 14)=2.510$, $P=0.149$], but it dramatically elevated brain 5-HIAA levels [$F(1, 14)=3.165$, $P=0.028$], resulting in a slight increase in the 5-HIAA/5-HT ratio [$F(1, 14)=0.133$, $P=0.720$]. Icariin exhibited a tendency towards an increase in brain NA levels [$F(4, 35)=0.854$, $P=0.500$]. On the other hand, no significant reduction of DA levels was observed after administration of icariin. Fluoxetine trended to increase 5-HT levels and decrease 5-HIAA levels [5-HT: $F(1, 14)=2.680$, $P=0.101$; 5-HIAA: $F(1, 14)=3.158$, $P=0.095$], resulting in a significant reduction in 5-HT turnover [$F(1, 14)=5.431$, $P=0.037$]. Amitriptyline, slightly but not significantly, increased brain HIAA levels [$F(1, 14)=0.335$, $P=0.571$], as well as 5-HT turnover [$F(1, 14)=1.799$, $P=0.199$]. This study did not show any significant changes in the NA and DA levels after fluoxetine or amitriptyline treatment [NA: $F(2, 21)=2.608$, $P=0.096$; DA: $F(2, 21)=0.571$, $P=0.573$].

3.5. Effects of icariin on serum CRF levels in stressed and normal mice

The effects of icariin, fluoxetine and amitriptyline after 21-consecutive-day treatments on serum CRF levels in stressed and normal mice were showed in Fig. 5. Swim stress procedure evoked a significant increase in serum CRF levels of saline-treated mice [$F(1, 22)=15.616$, $P=0.001$]. Icariin slightly but significantly, reduced the swim stress-induced increase in serum CRF levels, resulting in a significant effects of Stress [$F(1, 90)=12.549$, $P=0.001$] and Treatment × Stress interac-

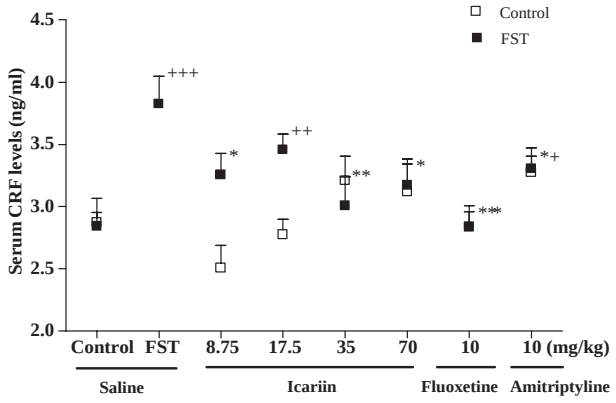

Fig. 5. Effects of icariin (8.75, 17.5, 35 and 70 mg/kg), fluoxetine (10 mg/kg) and amitriptyline (10 mg/kg) on serum CRF in stressed and normal in mice. They were orally administrated for 21 consecutive days, respectively. The data were given as the mean±SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared to the stressed animals (vehicle+saline). $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ when compared to the normal animals (vehicle+saline), respectively. The numbers of animals in stressed and normal groups were 12 and 8, respectively.

tion [$F(6, 126)=3.041$, $P=0.008$], and nonsignificant Treatment effect [$F(6, 126)=2.139$, $P=0.053$]. The maximal effect was obtained at 35 mg/kg [$F(1, 22)=6.501$, $P=0.018$]. Treatment with fluoxetine significantly prevented the swim stress-induced changes in serum CRF levels of mice [$F(1, 22)=15.253$, $P=0.001$]. Amitriptyline, also largely, but not completely, attenuated the swim stress-induced rise in serum CRF levels [$F(1, 22)=4.603$, $P=0.043$].

In unstressed animals, only an insignificant tendency towards a decrease in serum CRF levels was observed after icariin treatment at the lowest dose [$F(1, 14)=1.920$, $P=0.188$]. Conversely, icariin at higher doses exhibited a tendency to increase CRF levels [35 mg/kg: $F(1, 14)=1.457$, $P=0.247$; 70 mg/kg: $F(1, 14)=0.573$, $P=0.461$]. This study showed no changes in serum CRF levels after fluoxetine treatment [$F(1, 14)=0.023$, $P=0.882$]. Amitriptyline slightly but not significantly increased the CRF levels [$F(1, 14)=2.122$, $P=0.167$].

4. Discussion

The present study demonstrates that administration of icarrin has antidepressant-like activity in the FST and the TST. This behavioural effect was accompanied by icariin-induced modulation of both central neurochemical and the HPA axis response to the stress of FST exposure in mice.

In the mouse FST and FST, icariin produced a marked reduction in immobility time when orally administered at least for 14 and 7 consecutive days, respectively. However, clinically effective antidepressant fluoxetine and amitriptyline significantly reduced immobility time in the mouse FST and TST at all the dosed points. These findings indicated that the behavioural effects of icariin might be different from that of fluoxetine and amitriptyline.

Exposure to the FST led to a significant elevation of brain MAO-A and MAO-B activities in mice, which was in good agreement with our previous findings with ICR mice (Chen et al., 2005). It is well suggested that MAO-A act preferentially on 5-HT and NA and break down 5-HT to 5-HIAA, while DA is metabolized equally by MAO-A and MAO-B. The increases observed in brain MAO-A and MAO-B activities could consequently decrease these monoamine neurotransmitter levels in brain after the stress. Interestingly, in this study, it was found that the stress of FST procedure produced the reductions in brain 5-HT, 5-HIAA and NA concentrations and did not alter the DA levels. Some studies showed that the FST increased the levels of brain 5-HT, DA and their metabolites in male BALB/cA mice and Wistar rats (Miura et al., 1996; Linthorst et al., 2002). However, the NA concentration was reduced (Miura et al., 1996). On the other hand, the FST significantly elevated the ratio of 5-HIAA/5-HT, a usual indicator of serotonergic activity. Such an increase was in accordance with some results of Connor et al. (1997, 2000) obtained in the rat FST. These findings suggested that the stress of FST exposure accelerated the monoamine metabolism resulting from the enhancement of brain MAO-A and MAO-B activities.

The FST is a potent activator of the HPA axis system (Drossopoulou et al., 2004). A determinant role of endogenous CRF in behavioral responses was also suggested in other study carried out in the mouse FST (Garcia-Lecumberri and Ambrosio, 2000). Moreover, CRF, as a neurotransmitter or neuromodulator in brain, is under the control of monoamergic systems (Smialowska et al., 2001). Long-term elevation of CRF in rat brain resulted in hyporesponsivity of hippocampal 5-HT to stress (Linthorst et al., 1997). The reduction in hypothalamic NA and DA synthesis could be due to a direct effect of CRF treatment (De Pedro et al., 1998). In the present study, the FST induced a significant increase in serum CRF levels, which was concomitance with increased MAO-A and MAO-B activities and decreased monoamine neurotransmitter concentrations in mouse whole brain. These results support the notion that the effects of the FST stress on monoaminergic systems might be related, in this case, to the variation in the HPA axis function. The FST treatment used in our study was confirmed to be a good tool for mechanism elucidation of antidepressant drugs (Miura et al., 1996; Connor et al., 1997; Linthorst et al., 2002; Price et al., 2002; Drossopoulou et al., 2004; Chen et al., 2005).

Of great interest were the results showing that icariin not only inhibited MAO-A and MAO-B activities, but also clearly elevated the decreased brain monoamine neurotransmitter levels induced by the FST. After oral 21-consecutive-day administration, icariin ameliorated the FST-induced increases in brain MAO-A and MAO-B activities. This inhibitory activity for MAO was dependent on the doses of icariin. A tendency towards an inhibition of MAO-A activity in unstressed animals was founded after treatment with icarrin at 70 mg/kg. On the other hand, icariin at 35 and 70 mg/kg reversed the FST-induced decline of 5-HT levels, but not in 5-HIAA levels in brain, whereas, icariin at 17.5 and 8.75 mg/kg elevated both in the 5-HT and 5-HIAA levels. A depressed effect of icariin was observed for brain 5-HIAA/5-HT ratio in

the mouse FST. In unstressed mice following with icariin at 17.5 or 35 mg/kg, brain 5-HT levels were dramatically elevated. The former also significantly reduced the 5-HIAA/5-HT ratio. It was observed that icariin increased brain NA and DA levels in the FST. Higher doses of icariin trended to increase brain NA levels in the normal animals in the present study. Considering early reported experiments on the enhancements of 5-HT, 5-HIAA, NA and DA levels of hypothalamus in male old rats (Meng and Zeng, 1996) together with the data reported here, we support the hypothesis that icariin's antidepressant acts, at least in part, by inhibiting MAO-A and MAO-B activities and increasing the amount of these brain neurotransmitters in the FST animals, thus enhancing monoaminergic activity.

In the present study, fluoxetine produced a significant decrease in the 5-HIAA/5-HT ratio with increasing brain 5-HT levels and decreasing brain 5-HIAA levels in the mouse FST. These data were consistent with many previous reports concerning the ability of fluoxetine to reduce 5-HT turnover (Kirby and Lucki, 1997; Stenfors and Ross, 2002). As concerns fluoxetine, its modest NA-uptake inhibiting actions may contribute to the increase in brain NA levels. Fluoxetine tended to reverse the FST-induced decreases in brain NA and DA levels, but the effects were not statistically significant. These results were in accordance with another study (O'Shea et al., 2001). In line with amitriptyline nonselective ability to block 5-HT and NA reuptake, amitriptyline trended to increase 5-HT and 5-HIAA contents in mouse brain. However, it failed to alter the 5-HIAA/5-HT ratio change in the mouse FST.

Icariin at the doses used in this study were validated by several approaches, including measurement of behavior effects, MAO inhibitory activities and monoamine neurotransmitter increases in the mouse FST. Therefore, it might also be possible that icariin have a protective effect on the stress-evoked CRF changes. In the present, icariin elicited a marked diminution in serum CRF levels in the FST, suggesting that it could be beneficial in stress-related psychiatric disorders associated with an overactive CRF system.

Administration of fluoxetine diminished serum CRF production increase after the stress of FST exposure. Amitriptyline, also largely, but not completely, reversed the FST-induced rise in serum CRF levels. Consistent with these data were reports that chronic treatment with amitriptyline decreased CRF-R_1 receptor mRNA levels in the rat amygdala (Aubry et al., 1999), however, the data on fluoxetine effects on CRF synthesis and HPA axis activity were not unequivocal (Stout et al., 2002). It should note that methodological differences related to animal strain and its handling might have produced the disparate results.

In conclusion, the present study demonstrated that icariin had potential antidepressant-like activity. The mechanism involved in may be mediated by neurochemical and neuroendocrine systems. These findings suggested that icariin might be a potentially valuable drug for the treatment of depression. Preclinical and clinical studies are needed to verify such possibilities.

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