

## Ethanollic Extracts from *Curcuma longa* Attenuates Behavioral, Immune, and Neuroendocrine Alterations in a Rat Chronic Mild Stress Model

Xing XIA,<sup>a</sup> Ying PAN,<sup>a</sup> Wei-Yun ZHANG,<sup>a</sup> Guang CHENG,<sup>b</sup> and Ling-Dong KONG<sup>\*,a</sup>

<sup>a</sup>State Key Laboratory of Pharmaceutical Biotechnology, Immunobiological Laboratory, Nanjing University; Nanjing 210093, P. R. China; and <sup>b</sup>Nanjing Kanghai Pharmaceutical Company Limited; Nanjing 210061, P. R. China.

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The ethanollic extracts from the rhizome of *Curcuma longa* L. (turmeric), possesses a wide variety of biological activities related to the treatment and prevention of affective disorders. To study their antidepressant effects, the impacts of chronic mild stress (CMS) and of the subsequent administration of ethanollic extracts of *C. longa* were investigated. Male Sprague–Dawley rats subjected to the CMS procedure demonstrated increased serum interleukin-6 and tumor necrosis factor- $\alpha$  levels, as well as a reduction of natural killer cell activity in splenocytes. In addition, CMS-treated rats exhibited elevated corticotropin-releasing factor in serum and medulla oblongata and cortisol levels in serum, with no significant change in serum adrenocorticotropin hormone levels. The preferential behavior of reduction in sucrose intake was also observed. These findings indicate that the alterations in immune and hypothalamic-pituitary-adrenal (HPA) axis systems could participate in the behavioral response to the CMS procedure in animals. Administration of ethanollic extracts of *C. longa* largely reversed the above effects. These results demonstrate the antidepressant-like activity of ethanollic extracts of *C. longa* in the rat CMS model of depression, at least in part by improving the abnormalities in immune and the HPA axis functions.

**Key words** *Curcuma longa* L.; ethanollic extract; depression; chronic mild stress model; immune response; hypothalamic-pituitary-adrenal axis activity

Evidence suggests an association between depressive disorders and immune and neuroendocrine alterations.<sup>1)</sup> Cytokines, signaling molecules of the cellular immune system, profoundly influence neuroendocrine processes, thereby promoting affective disorders.<sup>2–4)</sup> Interleukin (IL) 6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), mainly derived from activated macrophages, are pleiotropic cytokines. Increased serum IL-6 levels have been observed in depressed patients, which were reversed after treatment with antidepressant drugs such as fluoxetine.<sup>5,6)</sup> Elevated circulating TNF- $\alpha$  might be associated with psychiatric illness.<sup>7)</sup> In addition to activation of cytokine secretion, a reduction in natural killer (NK) cell activity has also been reported in depressive patients.<sup>8)</sup> NK cell activity could be reversed by antidepressant drugs concomitant with clinical improvement in patients with depression.<sup>1,9)</sup> There are complex reciprocal relationships between immune and hypothalamic-pituitary-adrenal (HPA) axis function in depression.<sup>10,11)</sup> Elevated cerebrospinal fluid corticotropin-releasing factor (CRF) concentrations and altered regulation of adrenocorticotropin hormone (ACTH) and cortisol secretory activities have been observed in patients with major depression.<sup>12)</sup> Normalization of these levels occurred after successful antidepressant treatment with fluoxetine.<sup>13)</sup> On the other hand, some cytokines are known to be potent activators of the HPA axis and involved in the regulation of cortisol and ACTH secretion. It was important to note that animals displaying IL-6- and TNF- $\alpha$ -induced HPA axis sensitization showed signs of marked illness.<sup>14,15)</sup> In addition, NK cell activity is mediated by pro-inflammatory cytokines, mainly by IL-6 and TNF- $\alpha$ , which in depressed patients appear to be correlated with severity of the disease and hyperactivity of the HPA axis.<sup>11,16)</sup> These findings indicated that modulation of immune activities appeared to be in part under the control of the HPA axis.<sup>16)</sup>

The rhizomes of *Curcuma longa* L. (Zingiberaceae), com-

monly called turmeric, are widely used in food and medicine. Turmeric extracts have been shown to possess powerful antioxidant, anti-inflammatory, lipid-reducing, chemopreventive, immunomodulatory, and sedative actions.<sup>17,18)</sup> Current pharmacological studies show that they may represent a potential therapeutic approach to ameliorate the progression of Alzheimer's disease.<sup>19–21)</sup> Clinical results have recently suggested that premorbid major depressive disorder is associated with more severe cognitive deficits during the actual course of dementia.<sup>22)</sup> Our previous study demonstrated that aqueous extracts of *C. longa* were able to elicit a dose-dependent immobility reduction in the tail suspension test and the forced swimming test in mice, two animal models of depression.<sup>23)</sup> Curcumin, a major compound of the ethanollic extracts in turmeric, was found to attenuate the monoamine oxidase activity in C6 glial cells.<sup>24)</sup> In our continuing efforts to discover a suitable pharmacological therapy for treating depression, ethanollic extracts from *C. longa* was investigated in a rat model of chronic mild stress (CMS), which is implicated in the etiology of depression. The CMS paradigm appears, at first sight, to provide a relatively realistic animal model of the decreased response to reward (anhedonia) that characterizes depression, facilitating the study of concomitant changes in immune and neuroendocrine systems.<sup>25)</sup> Treatments with antidepressant drugs can block or reverse some parameters of CMS and depression. With regard to the immune system, we examined serum IL-6 and TNF- $\alpha$  levels and splenic NK cell activity. We also examined hormones related to the HPA axis such as CRF levels in serum and medulla oblongata and cortisol and ACTH levels in serum. Therefore, the aim of the present study was to explore the antidepressant effects of ethanollic extracts from *C. longa* on these concomitant biochemical parameters involved in the CMS model of depression in male Sprague–Dawley rats.

\* To whom correspondence should be addressed. e-mail: kongld@nju.edu.cn

## MATERIALS AND METHODS

**Chemicals** Fluoxetine hydrochloride tablets were purchased from Changzhou Siyao Pharmaceuticals Company Limited (Jiangsu province, P. R. China). Sodium lactate and bovine serum albumin were purchased from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Los Angeles, CA, U.S.A.). Nonidet P<sub>40</sub> (NP-40) was purchased from Fluka (Buchs SG, Switzerland). Nitroblue tetrazolium chloride (NBT) was obtained from BBI (Cocopaxi, CO, U.S.A.). Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and phenazine methosulfate (PMS) were from Amresco (Solon, OH, U.S.A.). Lactate dehydrogenase (LDH) substrate was composed of NBT, NAD<sup>+</sup>, PMS, and sodium lactate. All other reagents used were of analytical grade made in P. R. China.

**Plant Material** *C. longa* was botanically authenticated, and a voucher specimen has been deposited in the Herbarium of Nanjing University, P. R. China. The powdered rhizomes of *C. longa* (1000 g) was extracted three times with 95% EtOH (9.01) and kept for 48 h at room temperature (26–28 °C). The combined ethanolic extract was filtered and concentrated to dryness under reduced pressure at 50 °C. The weight of the extract obtained was 296.3 g, which was used for evaluation of antidepressant activity.

**Animals** Male Sprague–Dawley rats (Laboratory Animal Center, Jiangsu province, P. R. China) weighing 220–250 g were used in this experiment. Except as described below (see the CMS procedure), the animals were singly housed with food and water freely available and were kept on a controlled 12-h/12-h light/dark schedule with the lights on at 07:00 at a temperature of 22±2 °C. All experiments were approved by the Institutional Animal Care Committee of Nanjing University, or the China Council on Animal Care of Nanjing University.

**Chronic Mild Stress Model** The animals were first trained to consume a 1% sucrose solution intake. After a 3-d period of adaptation, the animals were divided into two subgroups, and sucrose solution intake baseline tests were performed (third weekly) over 14 d for all animals. These tests involved a 14-h period of food and water deprivation, followed by the offering of a sucrose solution for 1 h. Intake was measured by weighing pre-weighed bottles containing the sucrose solution at the end of each test. The intake was expressed in relation to the animal's body weight (g/kg). After the first phase (2 weeks), the animals were divided into two groups with similar average intake. One group was housed under normal conditions (non-stressed animals) and the other group was subjected throughout the experiment to CMS (CMS-treated animals). Subsequently, sucrose consumption was monitored under similar conditions, in 1-h tests (11:00–12:00 h) at weekly intervals for the next 12 weeks.

The CMS procedure was slightly modified from that previously described by others.<sup>25–27</sup> Each week consisted of one period (12 h) of paired caging, two periods (14, 18 h) of tilting cage (45°), two periods of water and food deprivation (14, 18 h), one 12-h period with a wet cage (200 ml of water in 100 g of sawdust bedding), and two periods (12 h each) of continuous light, three periods (6, 10, 12 h) of low-intensity

stroboscopic illumination (150 flashes/min), one 12-h period of intermittent illumination (2-h/2-h light/dark cycle), two periods of noise (6300-Hz tone, 10, 12 h). All of the stressors were applied individually and continuously, day and night.

Non-stressed rats were housed in a room separately from CMS animals. They were deprived of food and water for the 14 h preceding each sucrose test, but otherwise food and water were freely available in the home cage.

**Drug Administration** Both stressed and control animals were further divided into matched subgroups on the basis of sucrose intake scores following 5 weeks of stress. Subsequently, separate groups of non-stressed animals ( $n=7$  rats/per group) received oral vehicle (1 ml/kg) once daily, ethanolic extracts of *C. longa* at 35 and 70 mg/kg and fluoxetine at 7 mg/kg, respectively. Other separate groups of CMS-treated animals ( $n=7$  rats/group) received oral vehicle (1 ml/kg) once daily, *C. longa* extracts at 35 and 70 mg/kg and fluoxetine at 7 mg/kg, respectively. All drugs were suspended in a 0.9% normal saline solution and were orally administered once daily at 13:00 following the weekly sucrose intake test (approximately 1 h later) for the subsequent 7 weeks.

**Blood and Tissue Sampling** After the last sucrose intake test, all animals were left without any treatment until the following morning. To avoid fluctuations on cytokine and hormone levels due to circadian rhythms, blood samples were collected from the animals were bled at 09:00 and 10:00 on the day they were killed. Blood was collected on ice and separated in a refrigerated centrifuge at 4 °C. Serum was stored at –80 °C until assays were performed.

Immediately following decapitation, the medulla oblongata of all rats was dissected and placed into pre-weighed chilled plastic tubes treated with an enzyme inhibitor. The wet weight of the organs was then determined by subtracting the weight of the weight tray alone and expressed as wet weight. Tissue was dissociated using an ultrasonic cell disrupter. The dissociated samples were homogenized in 0.5 ml of 1 M HAc with protease inhibitors at 4 °C, then allowed to stand at room temperature for 1 h. Then 0.5 ml of 1 M NaOH was added and centrifuged at 8000 rpm for 5 min at 4 °C. Recovery ranged between 80% and 90% for extracted CRF. The supernatant was stored at –80 °C until assayed for CRF.

Following blood collection, the spleen was immediately removed under sterile conditions and pressed through a cell-dissociation sieve in DMEM for the NK cell activity assay.

**IL-6 and TNF- $\alpha$  Determination** Serum levels of IL-6 and TNF- $\alpha$  were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems), following the manufacturer's instructions. The minimum detectable concentrations were <14–36 pg/ml (mean 21 pg/ml) for IL-6 and <5 pg/ml for TNF- $\alpha$ .

**NK Cell Activity Assay** Splenic NK cell activity was measured using the LDH release assay as previously described,<sup>28</sup> using K562 cells as the target. Briefly, target tumor cells were washed with Hank's solution. The concentrations of K562 cells were adjusted to  $1.0 \times 10^5$ /ml with DMEM containing 10% FBS. Serial dilutions of splenocytes, used as effector cells, were pipetted into 96-well microplates in triplicate, with control wells containing either medium (for spontaneous release) or 1% NP-40 (for maximum release). Target cells ( $1.0 \times 10^4$ /0.1 ml) were added to all wells and in-

cubated for 2 h. The plate was centrifuged at 1000 rpm for 5 min, and 100  $\mu$ l of the supernatant was transferred to the corresponding wells of another microplate. Then 100  $\mu$ l of LDH substrate was added to each well. After 3 min, 50  $\mu$ l of 1 M HCl was added to each well to stop the reaction. Finally, the absorbance was recorded at 490 nm on a Bio-Rad microplate reader (Model 550).

The NK cell activity was expressed as the percentage specific lysis calculated by the following formula: % cytotoxicity =  $(E - S) / (M - S) \times 100$ , where  $E$  is the experimental release of LDH from the target cells incubated in the presence of spleen cells,  $M$  is the maximum release of LDH determined by lysing the target cells with 1% NP-40, and  $S$  is the spontaneous release of LDH from the target cells incubated in the absence of spleen cells.

**Hormone Assays** CRF levels in serum and medulla oblongata were measured using commercially available radioimmunoassay kits (Technique Center of Radioimmunity of the Navy, Beijing, P. R. China) in the Department of Immunoassay Inspection, Nanjing General Hospital, Nanjing Military Command, P. R. China. The sensitivity of the assay was 0.2 ng/ml. Intra- and inter-assay coefficients of variation for these assays was less than 8%.

Serum cortisol levels were determined using enzyme immunoassay (magnetic solid phase) kits (Beijing Bio-Ekon Biotechnology Company Limited, Beijing, P. R. China) in the Department of Endocrinology, Gu Lou Hospital Affiliated to the Medical College of Nanjing University, P. R. China. The sensitivity of the assay was 1.0 ng/ml. Intra- and inter-assay coefficients of variation were less than 4.85% and 6.08%, respectively.

Serum concentrations of ACTH were determined using commercially available radioimmunoassay kits (Technique Center of Radioimmunity of the Navy) in the Department of Immunoassay Inspection, Nanjing General Hospital, Nanjing Military Command. The sensitivity of the assay was 5 pg/ml. Intra- and inter-assay coefficients of variation were less than 6% and 12%, respectively.

**Statistical Analyses** Data are expressed as mean  $\pm$  S.E.M. calculated from the experimental groups of 7 rats. Statistical significance was determined using Student's  $t$ -test or analysis of variance with the Bonferroni test for *post hoc* comparison.  $p$  values of less than 0.05 were considered to be statistically significant.

## RESULTS

**Effects of Ethanolic Extracts of *C. longa* on Sucrose Intake** In the final baseline 1% sucrose intake test, all rats drank approximately 30 g/kg. After 5 weeks of CMS treatment, sucrose consumption was significantly lower in CMS-treated animals than in non-stressed controls. The intake was approximately 20 g/kg in the stressed animals, which continued until the end of the 7-week CMS.

Figure 1 illustrates the effects of the ethanolic extracts of *C. longa* and fluoxetine administration. The sucrose intake did not change significantly in the non-stressed control animals during this experiment. Non-stressed rats treated with ethanolic extracts at 35 and 70 mg/kg and fluoxetine at 7 mg/kg showed no marked variation in their preference for sucrose consumption (Fig. 1A).

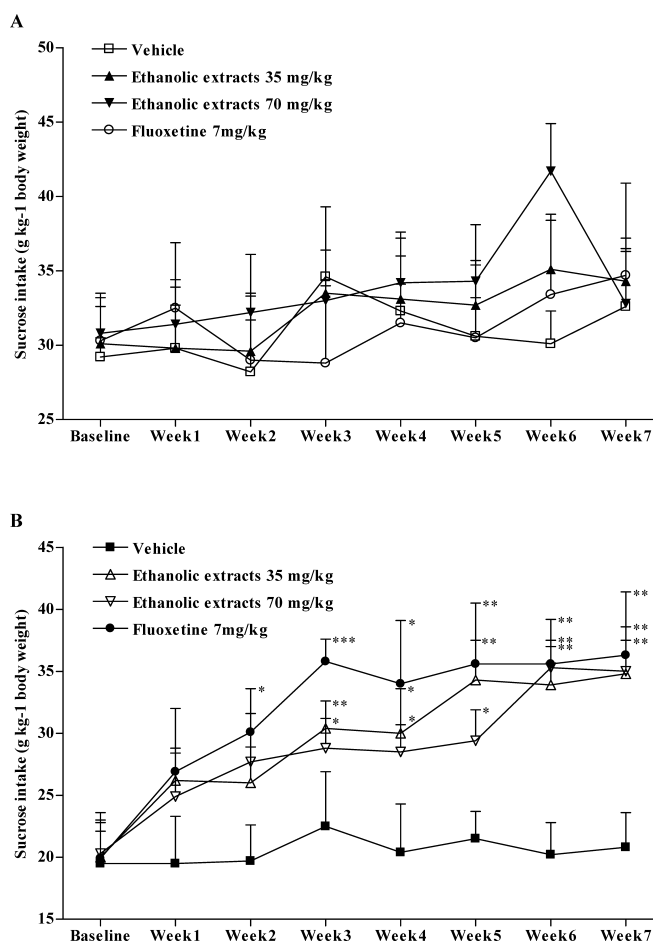


Fig. 1. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on Sucrose Intake in Non-stressed (A) and CMS-Treated rats (B)

Values are  $\pm$  S.E.M. No. of observation 7. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. drug-treated stressed animals at Week 0.

Compared with sucrose intake at week 0, ethanolic extracts at 35 and 70 mg/kg caused a gradual recovery of the sucrose intake after 3 weeks of treatment ( $p < 0.01$ ,  $p < 0.05$ , respectively), and the effects continued until the CMS procedure ceased. There was no significant difference between drug-treated stressed and vehicle-treated control animals. In contrast to the gradual onset of action of ethanolic extracts, fluoxetine at 7 mg/kg appeared to exert significant antidepressant effects after 2 weeks ( $p < 0.05$ ). At the end of 7 weeks and thereafter, the amounts of sucrose solution taken by stressed animals receiving the extracts at 35 and 70 mg/kg were consistently comparable with those of the fluoxetine-treated stressed group (Fig. 1B).

**Effects of Ethanolic Extracts of *C. longa* on Serum IL-6 Levels** As shown in Fig. 2, a significant increase in serum IL-6 levels in rats exposed to the CMS procedure was observed ( $p < 0.05$ ). The administration of ethanolic extracts of *C. longa* at 35 mg/kg and fluoxetine at 7 mg/kg prevented the stress-induced increase in serum IL-6 levels ( $p < 0.01$ ,  $p < 0.05$ ), although the extract at the dose of 70 mg/kg resulted in a small but no significant decrease in serum IL-6 levels. In the non-stressed groups, ethanolic extracts at 35 mg/kg appeared to elevate serum IL-6 levels, although there was no significant difference compared with the normal control group. In contrast, fluoxetine significantly elevated

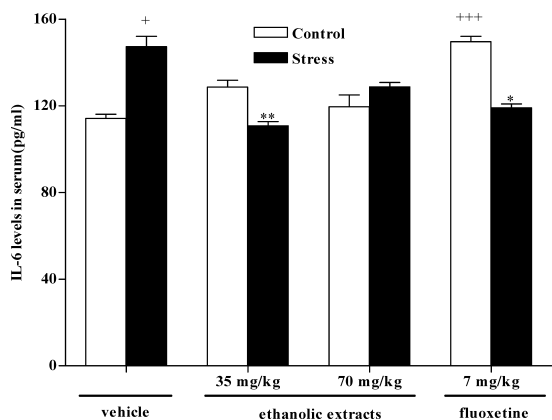


Fig. 2. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on IL-6 Levels in Serum in Non-stressed and CMS-Treated Rats

Values are  $\pm$ S.E.M. No. of observation 7. \* $p$ <0.05 and \*\* $p$ <0.01 vs. the vehicle-treated stressed control. † $p$ <0.05 and ††† $p$ <0.001 vs. the vehicle-treated non-stressed control.

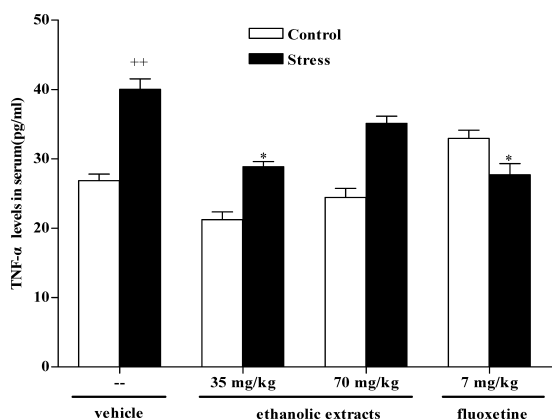


Fig. 3. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on TNF- $\alpha$  Levels in Serum in Non-stressed and CMS-Treated Rats

Values are  $\pm$ S.E.M. No. of observation 7. \* $p$ <0.05 vs. the vehicle-treated stressed control. †† $p$ <0.01 vs. the vehicle-treated non-stressed control.

serum IL-6 levels ( $p$ <0.001).

**Effects of Ethanolic Extracts of *C. longa* on Serum TNF- $\alpha$  Levels** As shown in Fig. 3, CMS induced a marked elevation in serum TNF- $\alpha$  levels ( $p$ <0.01). This effect was significantly attenuated by ethanolic extracts of *C. longa* at 35 mg/kg and fluoxetine at 7 mg/kg ( $p$ <0.05), but the extracts at the dose of 70 mg/kg had no significant effect on serum TNF- $\alpha$  levels. In the non-stressed groups, ethanolic extracts at 35 mg/kg resulted in a slight but no significant decrease in serum TNF- $\alpha$  levels. In contrast, fluoxetine showed a tendency to increase the levels.

**Effects of Ethanolic Extracts of *C. longa* on Splenic NK Cell Activity** The effects of ethanolic extracts of *C. longa* on splenic NK cell activity in non-stressed and CMS-treated rats are illustrated in Fig. 4. Comparisons of data obtained in the vehicle-treated stressed and non-stressed animals revealed that the CMS markedly reduced the NK cell activity ( $p$ <0.05). In the CMS-treated animals, ethanolic extracts at 35 and 70 mg/kg and fluoxetine at 7 mg/kg significantly enhanced splenic NK cell activity ( $p$ <0.05). It was noteworthy that after ethanolic extracts at 70 mg/kg treatment the NK cell activity in the stressed animals was greater than that of

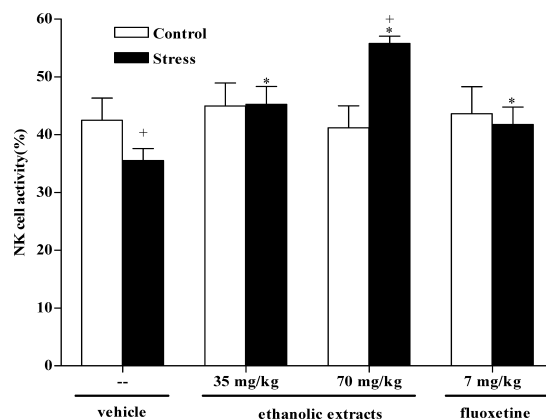


Fig. 4. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on NK Cell Activity in Splenocytes in Non-stressed and CMS-Treated Rats

Values are  $\pm$ S.E.M. No. of observation 7. \* $p$ <0.05 vs. the vehicle-treated stressed control. † $p$ <0.05 vs. the vehicle-treated non-stressed control.

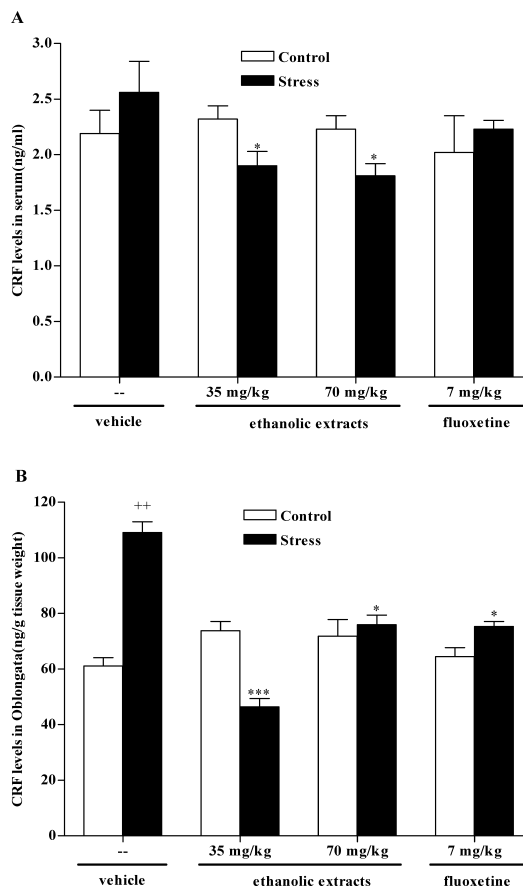


Fig. 5. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on CRF Levels in Serum (A) and Medulla Oblongata (B) in Non-stressed and CMS-Treated Rats

Values are  $\pm$ S.E.M. No. of observation 7. \* $p$ <0.05 and \*\*\* $p$ <0.001 vs. the vehicle-treated stressed control. †† $p$ <0.01 vs. the vehicle-treated non-stressed control.

the non-stressed controls ( $p$ <0.05). In non-stressed animals, ethanolic extracts of *C. longa* and fluoxetine did not significantly alter NK cell activity.

**Effects of Ethanolic Extracts of *C. longa* on CRF Levels in Serum and Medulla Oblongata** As shown in Fig. 5, the CMS-treated rats had slightly elevated serum CRF levels. Ethanolic extracts of *C. longa* at 35 and 70 mg/kg reduced

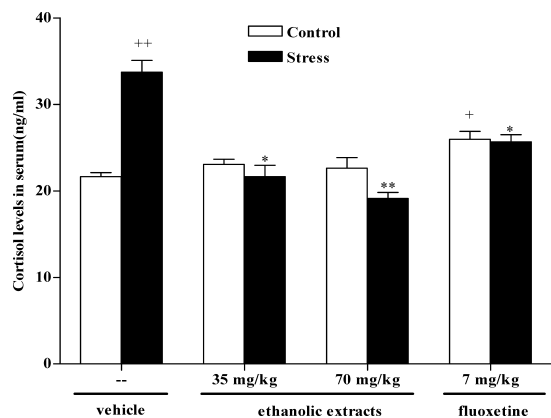


Fig. 6. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on Serum Cortisol Levels in Non-stressed and CMS-Treated Rats

Values are  $\pm$ S.E.M. No. of observation 7. \* $p < 0.05$  and \*\* $p < 0.01$  vs. the vehicle-treated stressed control.  $\dagger p < 0.05$  and  $\dagger\dagger p < 0.01$  vs. the vehicle-treated non-stressed control.

serum CRF levels of the stressed rats to lower than normal ( $p < 0.05$ ), but did not alter the levels of non-stressed animals (Fig. 5A). There was a slight but no significant decrease in serum CRF concentrations in both groups after fluoxetine administration.

There was a clear effect of the CMS procedure on CRF levels in medulla oblongata ( $p < 0.01$ ) (Fig. 5B). Ethanolic extracts at 35 and 70 mg/kg and fluoxetine at 7 mg/kg significantly inhibited the CMS-induced increase ( $p < 0.001$ ,  $p < 0.05$ , respectively). The extract at the dose of 35 mg/kg decreased the elevated CRF levels in medulla oblongata to values below the normal levels. A slight increase in CRF levels in medulla oblongata was observed after the administration of ethanolic extracts of *C. longa* and fluoxetine in the non-stressed rats.

**Effects of Ethanolic Extracts of *C. longa* on Serum Cortisol Levels** As shown in Fig. 6, CMS consistently increased serum cortisol levels relative to those in the non-stressed control rats ( $p < 0.01$ ). Ethanolic extracts of *C. longa* at 35 and 70 mg/kg significantly reversed the change to the normal levels ( $p < 0.05$ ,  $p < 0.01$ , respectively). Fluoxetine at 7 mg/kg also reduced serum cortisol levels ( $p < 0.05$ ), but did not return the elevated values to the normal levels. In the non-stressed groups, ethanolic extracts failed to affect the levels. Fluoxetine markedly elevated serum cortisol levels in the non-stressed rats ( $p < 0.05$ ).

**Effects of Ethanolic Extracts of *C. longa* on Serum ACTH Levels** As shown in Fig. 7, the serum ACTH levels of groups treated with the CMS procedure were not significantly increased compared with those of the non-stressed controls. Ethanolic extracts of *C. longa* did not change the levels in the two groups. However, fluoxetine at 7 mg/kg tended to increase serum ACTH levels in the non-stressed animals ( $p < 0.05$ ).

## DISCUSSION

The important observation in the present study was that sucrose intake reduction induced by CMS was related to specific alterations in immune and neuroendocrine parameters in male Sprague–Dawley rats and that they were reversed with chronic ethanolic extracts of *C. longa* administration. The

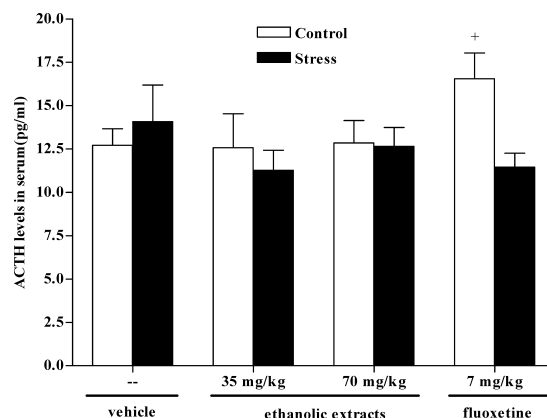


Fig. 7. Effects of Ethanolic Extracts from *C. longa* and Fluoxetine on Serum ACTH Levels in Non-stressed and CMS-Treated Rats

Values are  $\pm$ S.E.M. No. of observation 7.  $\dagger p < 0.05$  vs. the vehicle-treated non-stressed control.

results of the present study demonstrated that in comparison with normal animals, rats suffering the CMS procedure had significantly lower sucrose intake, which increased to the normal control values after ethanolic extracts of *C. longa* treatment. Furthermore, the extracts did not alter the intake in normal animals. As previously noted, *C. longa* was active in preclinical mice models of behavioral depression with effects comparable to those of known antidepressants.<sup>23)</sup> Thus the behavioral results from this current study confirmed and extended previous findings that chronic administration of the ethanolic extracts of *C. longa* had antidepressant-like effects in the CMS model of depression in rats.

CMS elicited significant elevations in serum levels of IL-6 and TNF- $\alpha$  and a marked reduction in splenic NK cell activity, which was in agreement with most published reports.<sup>3,7,14,27)</sup> Many researchers have reported that IL-6 administration activities the HPA axis and TNF- $\alpha$  also enhances HPA activation.<sup>14–16)</sup> In the present study, CMS-treated rats were characterized by higher CRF levels in serum and medulla oblongata, accompanied by significantly increased serum cortisol levels. The available data are consistent with the proposition that alterations of the HPA axis are related to depressive illness.<sup>12,29)</sup> The correlation of high IL-6 and TNF- $\alpha$  levels with CRF and cortisol levels in the CMS model in male Sprague–Dawley rats confirms the stimulatory effects of IL-6 and TNF- $\alpha$  on the HPA axis.

Our present results confirmed that the ethanolic extracts of *C. longa* prevent the CMS-induced increases in serum IL-6 and TNF- $\alpha$  levels, indicating that the effects of the ethanolic extracts on cytokine biosynthesis may contribute to their putative antidepressant properties. On the other hand, enhancement of NK cell activity was also observed in the CMS model of rats treated with ethanolic extracts of *C. longa*. These findings suggest that IL-6 and TNF- $\alpha$  levels and NK cell activity could be pharmacologically augmented by the ethanolic extracts of *C. longa* in the CMS-treated animals with immune function. In addition, the present finding of reduced serum IL-6 and TNF- $\alpha$  levels in CMS rats treated with fluoxetine at 7 mg/kg was in line with the data of Kubera *et al.*,<sup>30)</sup> who directly evaluated immune function based on the ability of splenocytes to produce cytokines. In the non-stressed rats, fluoxetine significantly increased serum

IL-6 levels, and a small but no significant increase in serum TNF- $\alpha$  levels was also observed. Recently, it has been reported that a combination of fluoxetine and 1-5-hydroxytryptophan increased the production of IL-6, and fluoxetine did not affect TNF- $\alpha$  production.<sup>31)</sup> In contrast, clinical researchers found that fluoxetine did not alter the basal levels of IL-6 and TNF- $\alpha$ , in spite of the improvement of mental disorders.<sup>5,32)</sup>

The ethanolic extracts significantly reduced the CMS-induced increase in CRF levels in serum and medulla oblongata to lower than normal and lowered elevated serum cortisol levels to the normal levels. These results suggested that the ethanolic extracts of *C. longa* exerted concerted effects on multiple elements of the HPA axis, manifesting functionally as a reduced neuroendocrine responsiveness to the CMS procedure in male Sprague–Dawley rats. It is worth noting that the ethanolic extract-induced decrease in levels of IL-6 and TNF- $\alpha$  and increased activity of NK cell were positively correlated with normality of the HPA axis system activity in the CMS model of depression in male Sprague–Dawley rats. Fluoxetine also reduced CRF levels in medulla oblongata and cortisol levels in serum in the CMS-treated rats, but did not return these levels to normal. There was no change in serum CRF levels in stressed animals after fluoxetine treatment. It was reported that fluoxetine decreased CRF messenger RNA in rat brain.<sup>33)</sup> There was no effect of fluoxetine administration on basal, median peak of the CRF concentration on depletion of the peptide after chronic stress.<sup>13)</sup> Based on our and other reports, it was plausible that a blunted effect of stress on CRF and cortisol levels may contribute to normalization of the HPA axis activity by fluoxetine observed in the CMS-treated rats. In addition, in non-stressed rats, fluoxetine produced a marked elevation of serum cortisol and ACTH levels. A small but no significant change in CRF levels in medulla oblongata was also observed after fluoxetine administration. These results support the early reports that a single dose of 80 mg/kg fluoxetine induced a slight increase in cortisol secretion when compared with placebo in an acute endocrine challenge test,<sup>34)</sup> and fluoxetine caused a significant increase in plasma ACTH levels as well as a significant increase in CRF concentrations in the hypophysial portal plasma.<sup>35)</sup>

Cyclooxygenase-2 (COX-2) is involved in the regulation of the immune system and in inflammation in the central nervous system via the effects of prostaglandins, in particular prostaglandin E<sub>2</sub>.<sup>36)</sup> Cytokines and stressors may increase blood-brain barrier permeability by increasing inflammatory factors, such as COX-2. TNF- $\alpha$  was reported to increase COX-2 expression as well as prostaglandin release at cerebral endothelial capillaries. These mediators are suggested to be responsible for the altered synthesis/activity of other cytokines and neurohormones. For example, increased prostaglandin release is involved in the stimulation of the HPA axis.<sup>37–39)</sup> It was observed that PGE<sub>2</sub> levels in blood and cerebrospinal fluid are increased in depressed patients.<sup>40)</sup> Cyclooxygenase blockers were found to suppress prostaglandin synthesis in brain structures involved in the regulation of the HPA axis activity.<sup>41,42)</sup> Recently, COX-2 inhibitors have been suggested to show beneficial effects in psychiatric disorders.<sup>43)</sup> Interestingly, curcumin, a major compound in turmeric, also has antiinflammatory and

immunomodulatory effects.<sup>17,18)</sup> One of mechanisms of curcumin is suggested to be due to COX-2 inhibition.<sup>44,45)</sup> Consequently, the hypothesis that the ethanolic extracts of *C. longa* act as a COX-2 inhibitor which, by lowering the concentration of inflammatory prostaglandins in the brain, reduces the detrimental impact of proinflammatory cytokine IL-6 and TNF- $\alpha$  changes and then normalizes the activity of the HPA axis, is the rationale for the development of antidepressant drugs. Based on these suggestions, the ethanolic extracts of *C. longa* have been investigated as a possible therapeutic approach in the treatment of depression.

In conclusion, the administration of the ethanolic extracts of *C. longa* largely reversed the CMS-induced reduction in sucrose intake associated with corresponding elevations in serum IL-6, TNF- $\alpha$ , and cortisol levels, as well as CRF levels in serum and medulla oblongata, and decreases in splenic NK cell activity. The enhancement of sucrose intake recovery response to the CMS procedure resulted at least in part from regulation of the abnormalities in immune and the HPA axis functions by the ethanolic extracts of *C. longa*. The potential of the ethanolic extracts of *C. longa* for human use as an antidepressant agent is worthy of exploration in the future.

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