

## Bidirectional regulation of bakuchiol, an estrogenic-like compound, on catecholamine secretion



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### ARTICLE INFO

#### Article history:

Received 1 August 2013

Revised 31 October 2013

Accepted 1 November 2013

Available online 11 November 2013

#### Keywords:

Bakuchiol

Catecholamine

Dual effects

Antidepressant-like effect

Anti-stress

### ABSTRACT

Excess or deficiency of catecholamine (CA) secretion was related with several diseases. Recently, estrogen and phytoestrogens were reported to regulate the activity of CA system. Bakuchiol is a phytoestrogen isolated from the seeds of *Psoralea corylifolia* L. (Leguminosae) which has been used in Traditional Chinese medicine as a tonic or aphrodisiac. In the present study, bovine adrenal medullary cells were employed to investigate the effects and mechanisms of bakuchiol on the regulation of CA secretion. Further, its anti-depressant like and anti-stress effects were evaluated by using behavioral despair and chronic immobilization stress models. Our results indicated that bakuchiol showed bidirectional regulation on CA secretion. It stimulated basal CA secretion in a concentration dependent manner ( $p < 0.01$ ), while it reduced 300  $\mu\text{M}$  acetylcholine (ACh) ( $p < 0.01$ ), 100  $\mu\text{M}$  veratridine (Ver) ( $p < 0.01$ ) and 56 mM  $\text{K}^+$  ( $p < 0.05$ ) induced CA secretion, respectively. We also found that the stimulation of basal CA secretion by bakuchiol may act through estrogen-like effect and the JNK pathway in an extra-cellular calcium independent manner. Further, bakuchiol elevated tyrosine hydroxylase Ser40 and Ser31 phosphorylation ( $p < 0.01$ ) through the PKA and ERK1/2 pathways, respectively. Bakuchiol inhibited ACh, Ver and 56 mM  $\text{K}^+$  induced CA secretion was related with reduction of intracellular calcium rise. In vivo experiments, we found that bakuchiol significantly reduced immobilization time in behavioral despair mouse ( $p < 0.05$  or  $0.01$ ), and plasma epinephrine (E) and norepinephrine (NE) levels in chronic immobilization stress ( $p < 0.05$ ). Overall, these results present a bidirectional regulation of bakuchiol on CA secretion which indicated that bakuchiol may exert anti-stress and the potential anti-depressant-like effects.

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### Introduction

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nephrine (E) and dopamine (DA), are important neurotransmitters that mediate various physiological effects. Typically, the “fight or flight response” induced by acute stress (Cannon and Paz, 1911) is accompanying excess CA secretion of the sympathetic nervous system which elevates heart rate, blood pressure and blood glucose to help animals escape from threats. Either excess or deficiency of CA secretion disturbs physiological functions. Therefore, pharmacological agents that alter

### bidirectional regulation of CA secretion: potential therapeutic effects

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neural crest cells in the developing embryo. They share a common sympathoadrenal progenitor with sympathetic neurons. In these cells, rise of intercellular concentration of  $\text{Ca}^{2+}$  is a prerequisite for secretion and synthesis of CA. Acetylcholine (ACh) induced  $\text{Na}^+$  influx by nicotinic ACh receptor-ion channels and veratridine (Ver) induced  $\text{Na}^+$  influx by voltage-dependent  $\text{Na}^+$  channels increase intercellular concentration of  $\text{Ca}^{2+}$  by voltage-dependent  $\text{Ca}^{2+}$  channels; high  $\text{K}^+$  directly gates voltage-dependent  $\text{Ca}^{2+}$  channels to increase  $\text{Ca}^{2+}$  influx without increasing  $\text{Na}^+$  influx (Wada et al., 1985). Because CA secretion mediated by stimulation of these ion channels in adrenal medullary cells was thought to be similar to that of norepinephrine (NE) and epinephrine (E) in the sympathetic neurons, bovine adrenal medullary cells have been widely used as a sympathetic model (Haass et al., 1997; Matsuda et al., 2008; Satoh et al., 2012).

Phytoestrogens are plant-derived compounds showing estrogen- or antiestrogen-like effects (Albertazzi and Purdie, 2002). Recent

Abbreviations: CA, catecholamine; NE, norepinephrine; E, epinephrine; DA, dopamine; DOPA, dihydroxyphenylalanine; TH, tyrosine hydroxylase.

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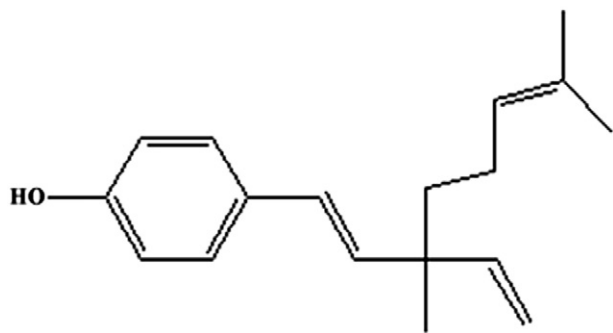


Fig. 1. Chemical structure of bakuchiol.

evidences demonstrate the regulation of estrogen and phytoestrogens on CA system. Estrogen receptors have been found in adrenergic neurons, cholinergic neurons and neuroendocrine cells (Haywood et al., 1999; Milner et al., 2001). Further evidences show that estrogen and phytoestrogens can regulate synthesis, secretion and degradation of CA. Estradiol benzoate elevates the mRNA expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of CA synthesis (Serova et al., 2002).  $17\beta$ -estradiol ( $E_2$ ) stimulates CA synthesis via activation of p44/42MAPK (Yanagihara et al., 2006) and decreases monoamine oxidase-A activity (Ma et al., 1993, 1995). Phytoestrogen like daidzein suppresses CA secretion at high concentrations while stimulating CA synthesis at low concentration (Liu et al., 2007). Resveratrol (3,4',5-trihydroxy-trans-stilbene), a natural polyphenolic compound found in grapes, berries and red wine, was reported to be an agonist for the estrogen receptor (Gehm et al., 1997). It suppresses CA secretion and synthesis induced by ACh (Shinohara et al., 2007). Cimicifugoside, a triterpene glycoside from black cohosh, possesses a steroid backbone structure, was called phytoestrogen. It did not affect the KCl-induced secretion but inhibited a nicotinic ACh receptor (nAChR) agonist 1,1-dimethyl-4-

phenylpiperazinium iodide (DMPP) induced CA secretion in bovine chromaffin cells (Woo et al., 2004).

Bakuchiol (the structure was showed in Fig. 1) is a prenylated phenolic monoterpene isolated from the seeds of *Psoralea corylifolia* L. (Leguminosae) which has been broadly used clinically in Traditional Chinese medicine. Kim KA reported that  $10\ \mu\text{M}$  bakuchiol had protective effects against oxidative stress (Kim et al., 2013). Bakuchiol also exerts antibacterial (Hsu et al., 2009), anti-inflammation (Choi et al., 2010; Ferrandiz et al., 1996), anti-tumor (Chen et al., 2010; Yang et al., 2010), immunosuppressive (Chen et al., 2008), and hepatoprotective effects (Cho et al., 2001; Park et al., 2007) and hypoglycemic activity (Krenisky et al., 1999). In addition, our group recently reported that bakuchiol possesses phytoestrogenic activity (Xin et al., 2010) which explains its effect in reducing bone loss in ovariectomized Sprague–Dawley rats (Lim et al., 2009). Meanwhile, bakuchiol analogs inhibit monoamine transporters and regulate monoaminergic functions (Zhao et al., 2008), suggesting a potential interaction of bakuchiol and CA. In the present study, we speculated that phytoestrogen bakuchiol may regulate CA secretion and this action may exert anti-depression or anti-stress activity. We employed chromaffin cells to investigate the effect of bakuchiol on the regulation of CA secretion. To examine the in vivo mental effects of bakuchiol, forced swim test (FST), tail suspension test (TST) and immobilization stress test (IMST) were employed in mice.

## Methods

**Isolation and primary culture of bovine adrenal medullary cells.** Adrenal glands were obtained from the city slaughterhouse. Bovine chromaffin cells were isolated as described previously (Mao et al., 2009). All the cells from different bovines (both male and female) were mixed together before planting. Primary cells between 2 and 7 days of culture were used for experiments.

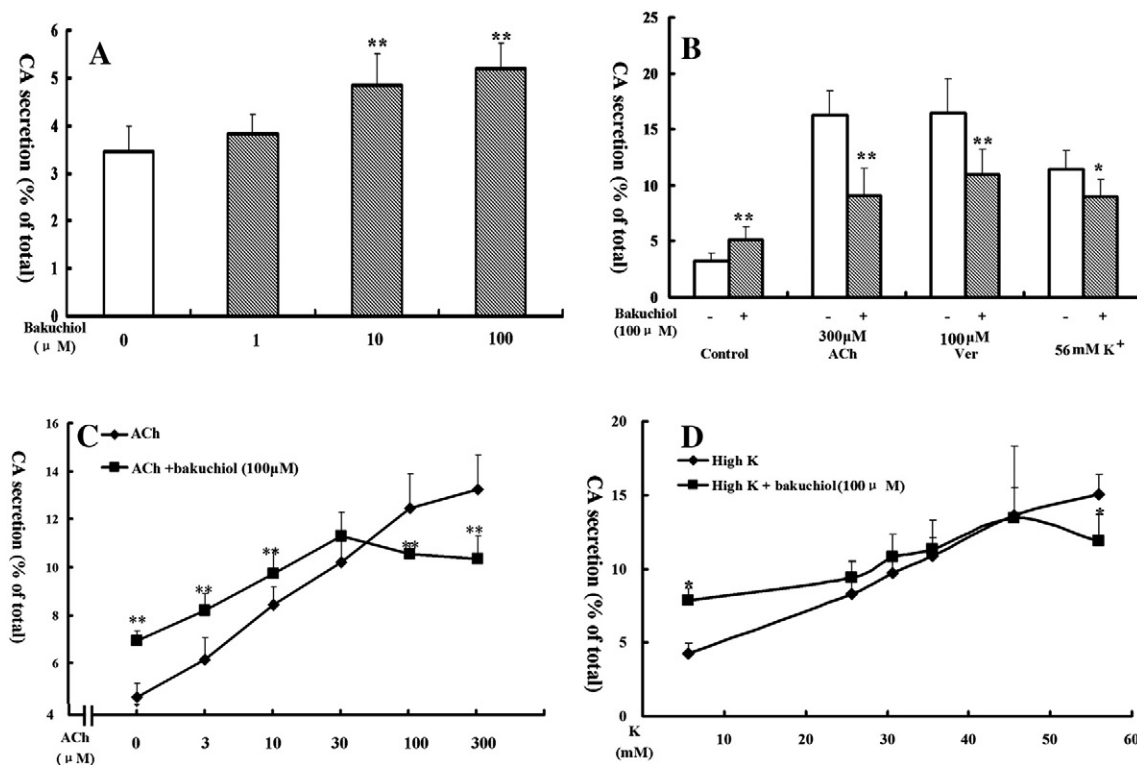
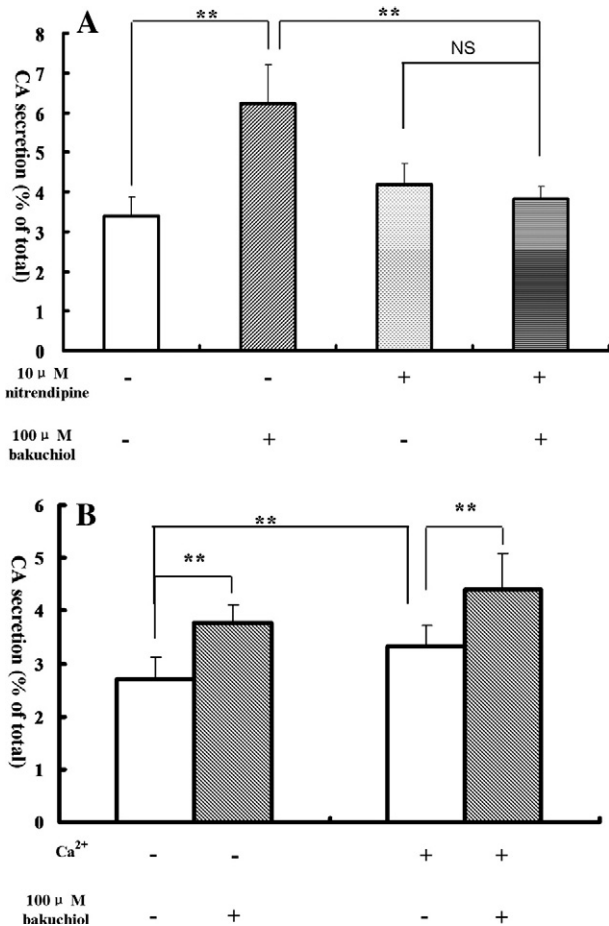


Fig. 2. Bidirectional effects of bakuchiol on CA secretion on bovine adrenal medullary cells. (A) Effects of 1–100  $\mu\text{M}$  bakuchiol on basal CA secretion; (B) effects of 100  $\mu\text{M}$  bakuchiol on various secretagogues induced CA secretion. (C) Effects of 100  $\mu\text{M}$  bakuchiol on CA secretion induced by various concentrations of ACh and (D) effects of 100  $\mu\text{M}$  bakuchiol on CA secretion induced by various concentrations of  $\text{K}^+$ . Data were expressed in means  $\pm$  SD based on three independent experiments in triplicate ( $n = 7\text{--}9$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ .



**Fig. 3.** Stimulation of bakuchiol on basal CA secretion on cultured bovine adrenal medullary cells. (A) The cells were treated with or without nitrendipine (10  $\mu$ M) in the presence or absence of bakuchiol (100  $\mu$ M) for 10 min at 37  $^{\circ}$ C ( $n = 6$  per group). (B) Cells were incubated with Ca<sup>2+</sup> (-) KRP or Ca<sup>2+</sup> (+) KRP in the presence or absence of bakuchiol (100  $\mu$ M) for 10 min at 37  $^{\circ}$ C ( $n = 8$  or 9 per group). CAs secreted into the medium were measured, and expressed as a percentage of the total CAs in the cells. Data were means  $\pm$  SD from three independent experiments in triplicate. \*\* $p < 0.01$ , NS means no significance.

**Drugs and reagents.** Oxygenated Krebs–Ringer phosphate (KRP) buffer was used in cell experiments in this study. Its composition is as follows (in mM): 154 NaCl, 5.6 KCl, 1.1 MgSO<sub>4</sub>, 2.2 CaCl<sub>2</sub>, 0.85 NaH<sub>2</sub>PO<sub>4</sub>, 2.15 Na<sub>2</sub>HPO<sub>4</sub> and 10 glucose, adjusted to a pH of 7.4. Reagents were obtained from the following sources: Eagle's minimum essential medium (MEM) and new born calf serum from Gibco (USA); collagenase from Nitta Zerachin (Japan); cytosine arabinoside, ACh, Ver, nitrendipine, U0126, PD98059 and venlafaxine from Sigma (St. Louis, MO, USA); primary antibodies of phospho-P44/42 MAPK (Erk1/2) (#9101), p44/42 MAPK (Erk1/2) (#4695), phospho-SAPK/JNK (#9251), SAPK/JNK (#9252), phospho-tyrosine hydroxylase (Ser 31) (#3370), phospho-tyrosine hydroxylase (Ser40) (#2791), and tyrosine hydroxylase (#2792) were from Cell Signaling Technology; lysis buffer (P0013B), enhanced BCA protein assay kit (P0010) and SDS-PAGE sample loading buffer (P0015) were from Beyotime Institute of Biotechnology (China); bakuchiol was isolated from *P. corylifolia* L. (Leguminosae) and identified by <sup>1</sup>H NMR method in our laboratory. Quantitative analysis of the test samples by liquid chromatography showed that the purity of bakuchiol is greater than 97.0%. For in vitro experiments, bakuchiol was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in a reaction medium (final concentration of bakuchiol was between 1  $\mu$ M to 100  $\mu$ M) before use with a final concentration of DMSO lower than 0.5%. Different secretagogues and agents were co-administered with bakuchiol, except when used alone. For in vivo experiments,

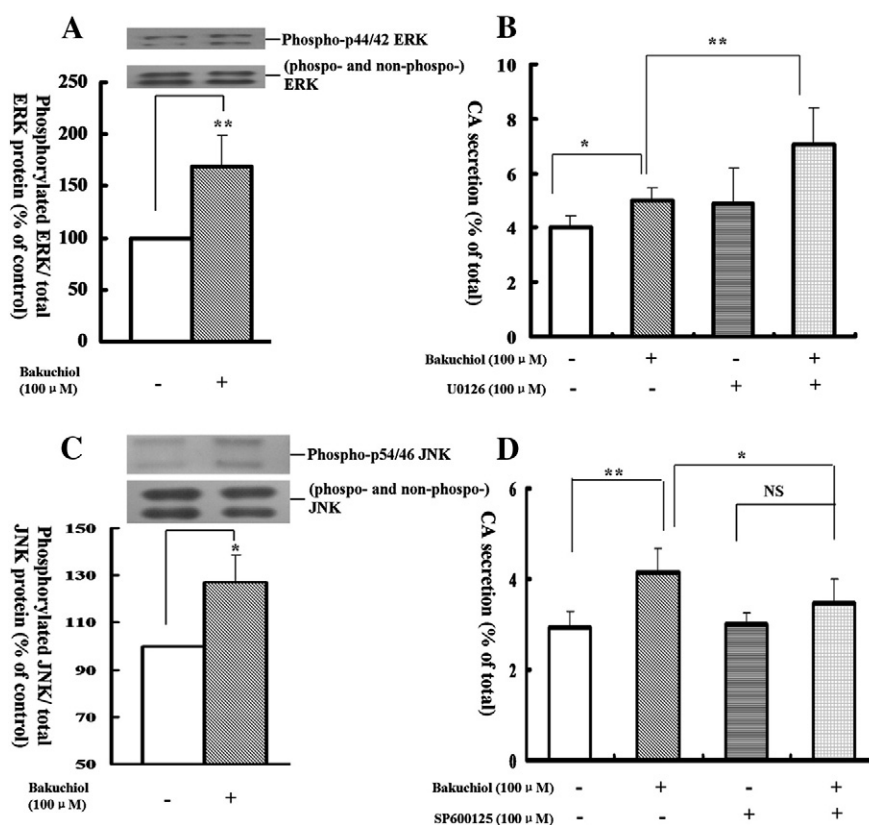
bakuchiol was dissolved in 0.2% sodium carboxymethyl cellulose at 1 mg/ml, 2 mg/ml and 4 mg/ml stock solutions. Venlafaxine was dissolved in 0.2% sodium carboxymethyl cellulose at 0.67 mg/ml as stock solution. The above stock solutions were administrated at 15 ml/kg body weight by intragastric (i.g.) route. Appropriate vehicle-treated groups (0.2% sodium carboxymethyl cellulose) were also assessed simultaneously.

**Detection of CAs secreted by cultured bovine adrenal medullary cells by HPLC-ECD.** To detect the effect of bakuchiol on CA secretion, the CAs in culture medium were detected by HPLC-ECD. Briefly, bovine adrenal medullary cells were buffered with oxygenated KRP and washed 3 times before experiments. After incubation with the indicated agents at 37  $^{\circ}$ C for 10 min, medium was transferred immediately to a test tube containing perchloric acid (PCA) (final concentration, 0.4 M). NE and E secreted into the medium were detected by a high-performance liquid chromatographic system coupled with an electrochemical detector (HPLC-ECD). The chromatographic separation system included RP C18 analytical column (5  $\mu$ m,  $\phi$ 4.6 mm  $\times$  250 mm) and an isocratic solvent system. The flow phase (1 l) contained: 100 ml acetonitrile, 100  $\mu$ l triethylamine, 13.6 g KH<sub>2</sub>PO<sub>4</sub>, 2.5 g Octanesulfonic acid sodium and 0.036 g EDTA, adjusted pH to 3.3 with H<sub>3</sub>PO<sub>4</sub>. The flow rate was 0.6 ml/min, column temperature 30  $^{\circ}$ C and sample injection volume 10  $\mu$ l. The detection was obtained using an electrochemical detector with two channels: one is at 0 mV and the other +300 mV.

**Measurement of intracellular Ca<sup>2+</sup> mobilization.** To examine the effects of bakuchiol on intracellular Ca<sup>2+</sup> concentration induced by different secretagogues, cells were seeded into black-walled clear-base 96-well plates (Greiner, USA) at a density of about 50,000 cells per well in culture media and grown for approximately 24 h in a 37  $^{\circ}$ C CO<sub>2</sub>-incubator. The cells were incubated with the Calcium 4 reagent (Molecular Devices, Sunnyvale, CA, USA) for 60 min in a 37  $^{\circ}$ C CO<sub>2</sub>-incubator. The Calcium 4 reagent-loaded cells were checked on a FlexStation III (Molecular Devices, Sunnyvale, CA, USA), to monitor fluorescence (ex = 485 nm, em = 525 nm) before and after the addition of bakuchiol (1–100  $\mu$ M). Bakuchiol was prepared in assay buffer with final DMSO concentration less than 0.5%. Intracellular Ca<sup>2+</sup> mobilization was measured as relative fluorescence units (RFU) and expressed as percentage of RFU at 0 s.

**Western blot.** The effects of bakuchiol on the activation of MAPK (ERK, JNK and p38) pathway and TH proteins were tested by Western blot analysis. After treatment, culture medium was discarded. The cells were washed twice with ice-cold KRP buffer, and lysed in lysis buffer. Protein concentration was measured using an enhanced BCA protein assay kit, and equal amounts of protein were mixed with 5 $\times$  SDS-PAGE sample loading buffer. Following heating at 95  $^{\circ}$ C for 5 min, proteins were subjected to 10% Tris-glycine gel and transferred electrophoretically to polyvinylidene difluoride membrane (Bio-Rad). Incubation with primary antibodies (1:1000) was performed overnight at 4  $^{\circ}$ C and then with horseradish peroxidase-labeled secondary antibodies (1:5000) for 1 h at room temperature. Antibody-positive band was visualized using enhanced chemiluminescence Western blot detection reagents. The band densities were measured by Quantity One software using VersaDoc imaging system (Bio-Rad). Data were expressed as a ratio of phosphorylated proteins to total proteins (phosphorylated-style plus non-phosphorylated-style).

**Animals.** Male ICR strain mice with body weight ranging 18–20 g (Tianjin Laboratory Animal Center, Certificate number SCKX-2010-0002) were used. Animals were group-housed under 12 h/12 h light/dark schedule and had free access to tap water and food. Ambient temperature and relative humidity were maintained at 24  $\pm$  1  $^{\circ}$ C temperature and 55  $\pm$  5% humidity, respectively. Mice were acclimatized to the lab for 7 days before experiments. Animal experiments were carried out



**Fig. 4.** Role of the MAPK pathway in bakuchiol-induced CA secretion on bovine adrenal medullary cells. (A) Western blot of ERK pathway phosphorylation ( $n = 6$  per group) induced by bakuchiol. (B) Effect of ERK pathway antagonist U0126 on CA secretion induced by bakuchiol ( $n = 8$  or 9 per group). (C) Western blot of JNK pathway phosphorylation ( $n = 6$  per group) induced by bakuchiol. (D) Effect of JNK pathway antagonist SP600125 on CA secretion induced by bakuchiol ( $n = 8$  or 9 per group). \* $p < 0.05$ , \*\* $p < 0.01$ , NS means no significance.

at 1 h after administration of bakuchiol or vehicle between 9:00 and 15:00. Animal handling procedures performed in this study were in accordance with NIH Guide for the Care and Use of Laboratory Animals.

**The forced swim test (FST).** FST was used to test effects of bakuchiol against acute stress. FST was performed following the method described previously (Kaster et al., 2007) with minor modifications. Venlafaxine, an antidepressant used in clinic, was used in this study as a positive control. Briefly, mice were divided into the following groups with  $n = 9$  or 10 in each group: vehicle (0.2% sodium carboxymethyl cellulose), venlafaxine (10 mg/kg of body weight) and bakuchiol (15, 30 or 60 mg/kg of body weight). A glass cylinder, 20 cm in height and 10 cm in diameter, was filled with 15 cm of water (23–25 °C). Mice were placed in water for 6 min and subsequently desiccated before being returned to their cages. The sessions were videotaped, and scored by two independent observers to calculate the accumulated immobility time during the last 4 min. The immobility behavior is defined as a mouse which does not make any active movements to escape from the tank and just keeps itself afloat in water.

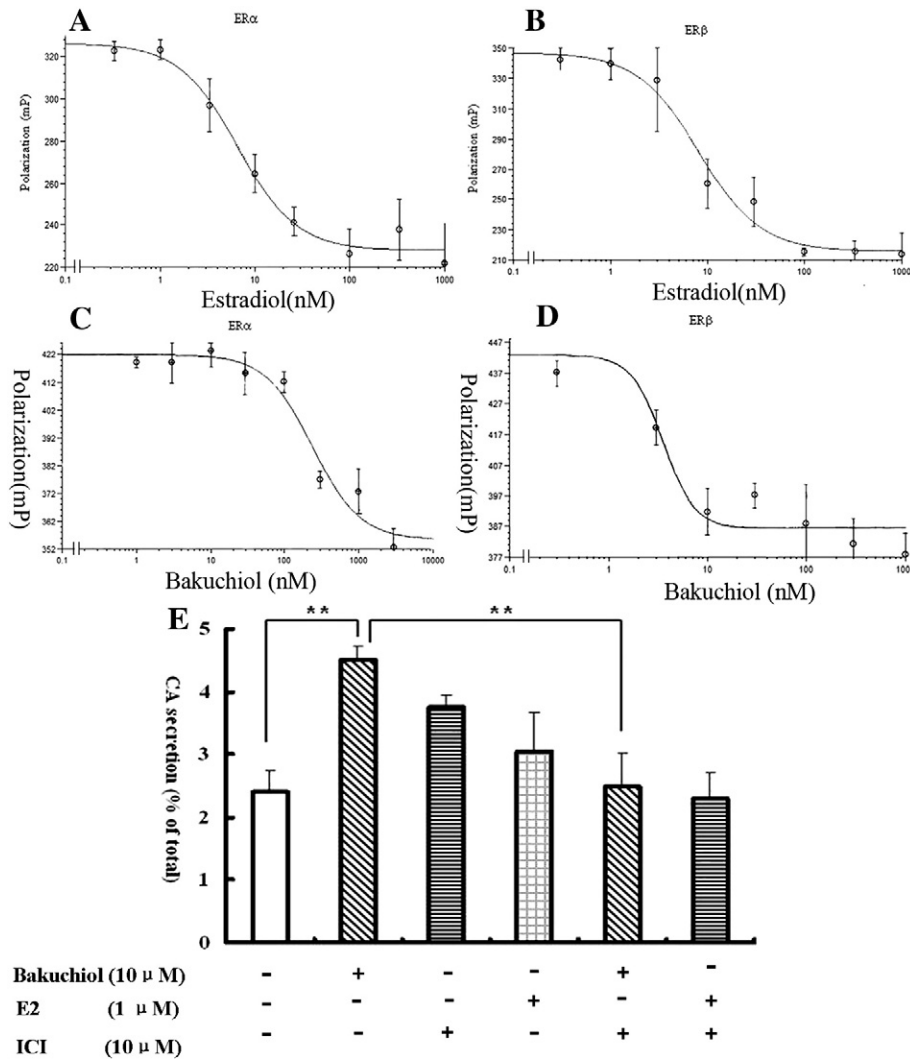
**The tail suspension test (TST).** TST was another model used to test the effects of bakuchiol on acute stress. It was performed following the method of Steru et al. (1985) with minor modifications. Briefly, mice were divided into the following groups with  $n = 9$  or 10 animals in each group: vehicle (0.2% sodium carboxymethyl cellulose), venlafaxine (10 mg/kg of body weight) and bakuchiol (15, 30 or 60 mg/kg of body weight). Mice were individually suspended by the tail using adhesive scotch tape on a pole above the floor and the total duration of immobility was calculated during a 6-min test. Data collected were expressed as a mean of total immobility time in the 6 min test.

**Open-field test.** Open field test was employed to detect the effects of bakuchiol on locomotor activities. It was performed as described previously (Brocardo et al., 2008a, 2008b) with minor modifications. Briefly, mice were divided into the following groups ( $n = 8$ –10 per group): vehicle (0.2% sodium carboxymethyl cellulose), venlafaxine (10 mg/kg of body weight) and bakuchiol (15, 30 or 60 mg/kg of body weight). Each mouse was placed individually into the center of the “open field” apparatus. Numbers of crossing and rearing were measured during the last 4 min in a 6-min observation period. Experiments were performed in the dark room and the apparatus was illuminated by a 60 W bulb positioned 1 m above the center of the circle.

**Chronic anti-stress test.** Chronic anti-stress test was employed to detect the effect of bakuchiol on stress induced CA elevation. It was performed as described previously (Kvetnansky and Mikulaj, 1970) with some modifications. Briefly, mice were divided into the following groups ( $n = 7$ –9 per group): vehicle (0.2% sodium carboxymethyl cellulose), stress (0.2% sodium carboxymethyl cellulose and immobilization stress) and bakuchiol (15, 30 or 60 mg/kg of body weight and immobilization stress). Mice were immobilized in the tubes for 1 h/day in 7 consecutive days to induce chronic stress. On the 7th day after chronic mild stress test, animals were euthanized by decapitation. Blood samples were collected and centrifuged at 3000  $\times$ g for 15 min at 4 °C. The serum samples collected were detected by HPLC-ECD analysis as described above.

**Statistical analysis.** Data were presented as mean  $\pm$  SD. Results of Western blots about MAPK were assessed using paired  $t$ -test. For chronic stress assessments, Student's  $t$  test was used to compare the effects between stress and control, and then one-way ANOVA followed by





**Fig. 5.** Estrogenic effect of bakuchiol on CA secretion on bovine adrenal medullary cells. Concentration curve of E<sub>2</sub> binding to (A) estrogen receptor  $\alpha$  (n = 5 per group) and (B) estrogen receptor  $\beta$  (n = 5 per group). Concentration curve of bakuchiol binding to (C) estrogen receptor  $\alpha$  (n = 5 per group) and (D) estrogen receptor  $\beta$  (n = 5 per group) and (E) effect of antagonist of estrogen receptor, ICI 162,780 (10  $\mu$ M) on bakuchiol (10  $\mu$ M) or E<sub>2</sub> (1  $\mu$ M) induced CA secretion for 24 h (n = 4–6 per group). \*\*p < 0.01.

Dunnnett's post hoc tests was conducted to identify differences among the groups between stress and bakuchiol. Other assessments were analyzed by using one-way ANOVA followed by Dunnnett's post hoc tests. The FST and TST data were blinded before review. Significance level was set at p < 0.05.

## Results

### Dual effects of bakuchiol on CA secretion in cultured bovine adrenal medullary cells

Bovine adrenal medullary cells were treated with different secretagogues in the presence or absence of 100  $\mu$ M bakuchiol. Bakuchiol alone significantly stimulated CA secretion (vs. baseline, p < 0.01). Co-treatment of 100  $\mu$ M bakuchiol with 300  $\mu$ M ACh, 100  $\mu$ M Ver or 56 mM K<sup>+</sup> for 10 min significantly inhibited CA secretion (p < 0.05 or 0.01) (Fig. 2A). 10  $\mu$ M and 100  $\mu$ M bakuchiol stimulated basal CA secretion (vs. baseline, p < 0.01, Fig. 2B).

100  $\mu$ M bakuchiol stimulated CA secretion induced by ACh at 3  $\mu$ M and 10  $\mu$ M, compared with 3  $\mu$ M and 10  $\mu$ M ACh alone (p < 0.01). 100  $\mu$ M bakuchiol significantly inhibited CA secretion induced by 100  $\mu$ M and 300  $\mu$ M ACh (p < 0.01, Fig. 2C). When 5.6 mM, 25.6 mM, 35.6 mM, 45.6 mM and 56 mM K<sup>+</sup> were used, 100  $\mu$ M bakuchiol

stimulated CA secretion induced by 5.6 mM K<sup>+</sup>, while it inhibited 56 mM K<sup>+</sup> induced CA secretion (p < 0.05, Fig. 2D).

### Stimulation of bakuchiol on basal CA secretion in cultured bovine adrenal medullary cells

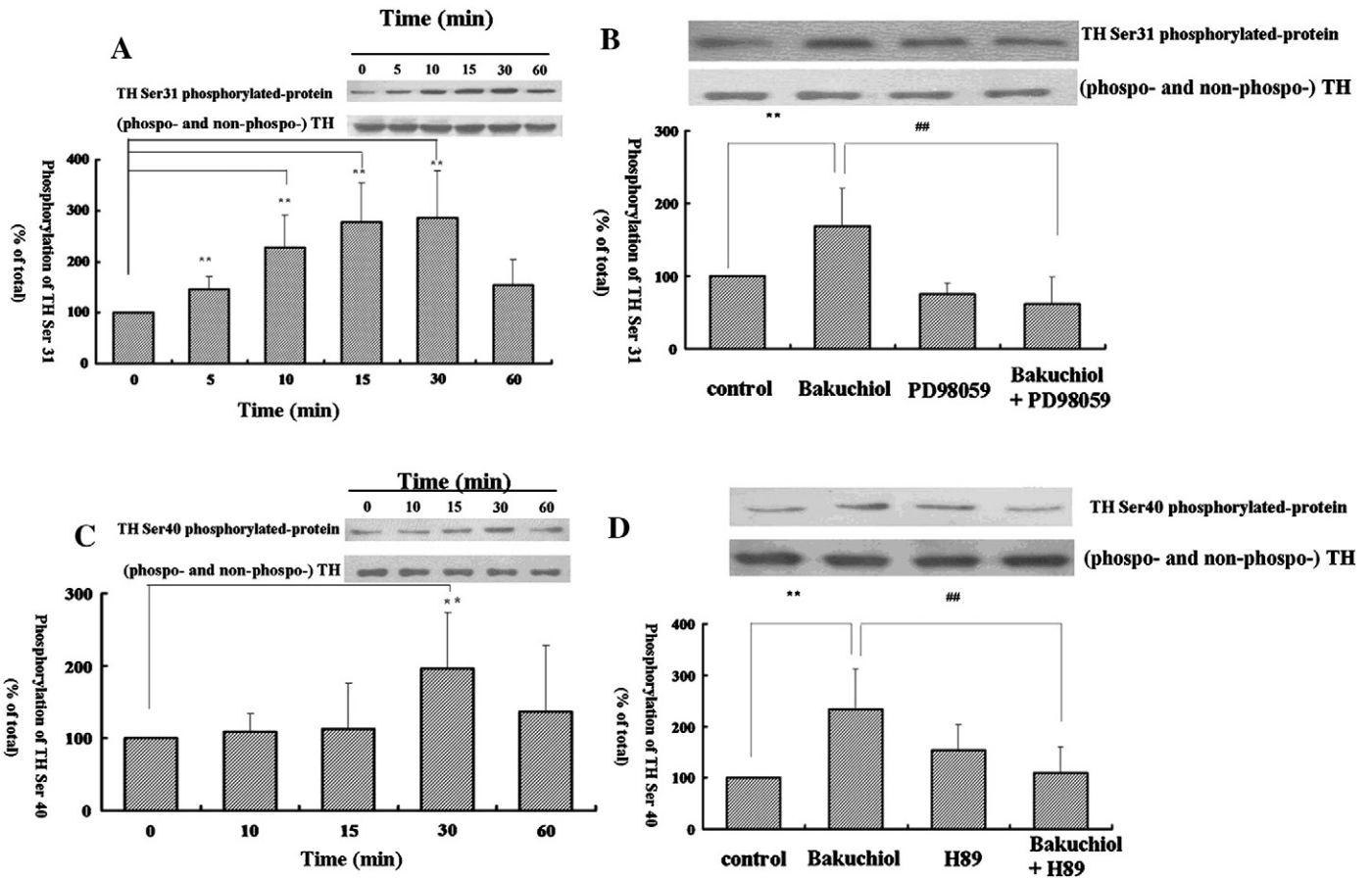
#### Stimulatory modes of bakuchiol on basal CA secretion in cultured bovine adrenal medullary cells

The effect of nitrendipine, a Ca<sup>2+</sup> channel blocker, on bakuchiol-induced CA secretion was measured. 10  $\mu$ M nitrendipine significantly attenuated bakuchiol-induced CA secretion compared with bakuchiol alone (p < 0.01), but there was no significant difference between nitrendipine and nitrendipine plus bakuchiol groups (p = 0.34, Fig. 3A).

We also evaluated that bakuchiol significantly stimulated the basal CA secretion in the presence or absence of extracellular Ca<sup>2+</sup> (p < 0.01, Fig. 3B).

#### The role of MAPK pathway activation in bakuchiol-induced catecholamine secretion

Results of Western blot showed that 100  $\mu$ M bakuchiol alone significantly promoted the p44/42 ERK pathway phosphorylation (Student's t test, p = 0.009, Fig. 4A). 100  $\mu$ M U0126, an antagonist of the ERK pathway, stimulated CA secretion induced by 100  $\mu$ M bakuchiol (p < 0.01,



**Fig. 6.** Effects of bakuchiol on the phosphorylation of tyrosine hydroxylase on bovine adrenal medullary cells. (A) Time course of TH Ser31 phosphorylation (n = 9 per group) induced by 100 μM bakuchiol. (B) Effect of ERK pathway on phosphorylation of TH Ser31 (n = 6 or 7 per group) induced by 100 μM bakuchiol. (C) Time course of TH Ser40 phosphorylation (n = 9 per group) induced by 100 μM bakuchiol. (D) Effect of PKA pathway on phosphorylation of TH Ser40 (n = 7 per group) induced by 100 μM bakuchiol. \*\*p < 0.01.

Fig. 4B). PD98059, another antagonist of ERK pathway, had the same effect as with U0126 (data not shown).

100 μM bakuchiol alone also significantly promoted the phosphorylation of the JNK pathway (Student's *t* test, *p* = 0.02, Fig. 4C). SP600125 (100 μM), an antagonist for the JNK pathway, significantly inhibited 100 μM bakuchiol induced CA secretion (*p* < 0.05) while there was no significant difference between SP600125 and SP600125 plus bakuchiol groups (*p* = 0.09, Fig. 4D). However, we found that bakuchiol did not affect the phosphorylation of the p38 pathway (data not shown).

*Bakuchiol stimulated CA secretion through estrogenic effect*

Fluorescence polarization method was used to investigate the affinity of E<sub>2</sub> and bakuchiol on ERα and ERβ. The IC<sub>50</sub> of E<sub>2</sub> for ERα and ERβ was 6.60 nM and 13.60 nM (Figs. 5A and B), respectively. The IC<sub>50</sub> of bakuchiol for ERα and ERβ was 241.00 nM and 3.52 nM (Figs. 5C and D), respectively. When ICI 182,780 (10 μM), an antagonist of estrogen receptor, were incubated with bakuchiol (10 μM) or E<sub>2</sub> (1 μM) for 24 h, CA secretion induced by bakuchiol was significantly decreased (*p* < 0.01, Fig. 5E).

*Effect of bakuchiol on the phosphorylation of tyrosine hydroxylase in bovine adrenal medullary cells*

Western blot assay showed that after incubation with 100 μM bakuchiol for 0–60 min, non-phosphorylated-tyrosine hydroxylase protein expression was not changed. Similar result was also observed when the cells were incubated with 10 μM bakuchiol for 24 h (data not shown).

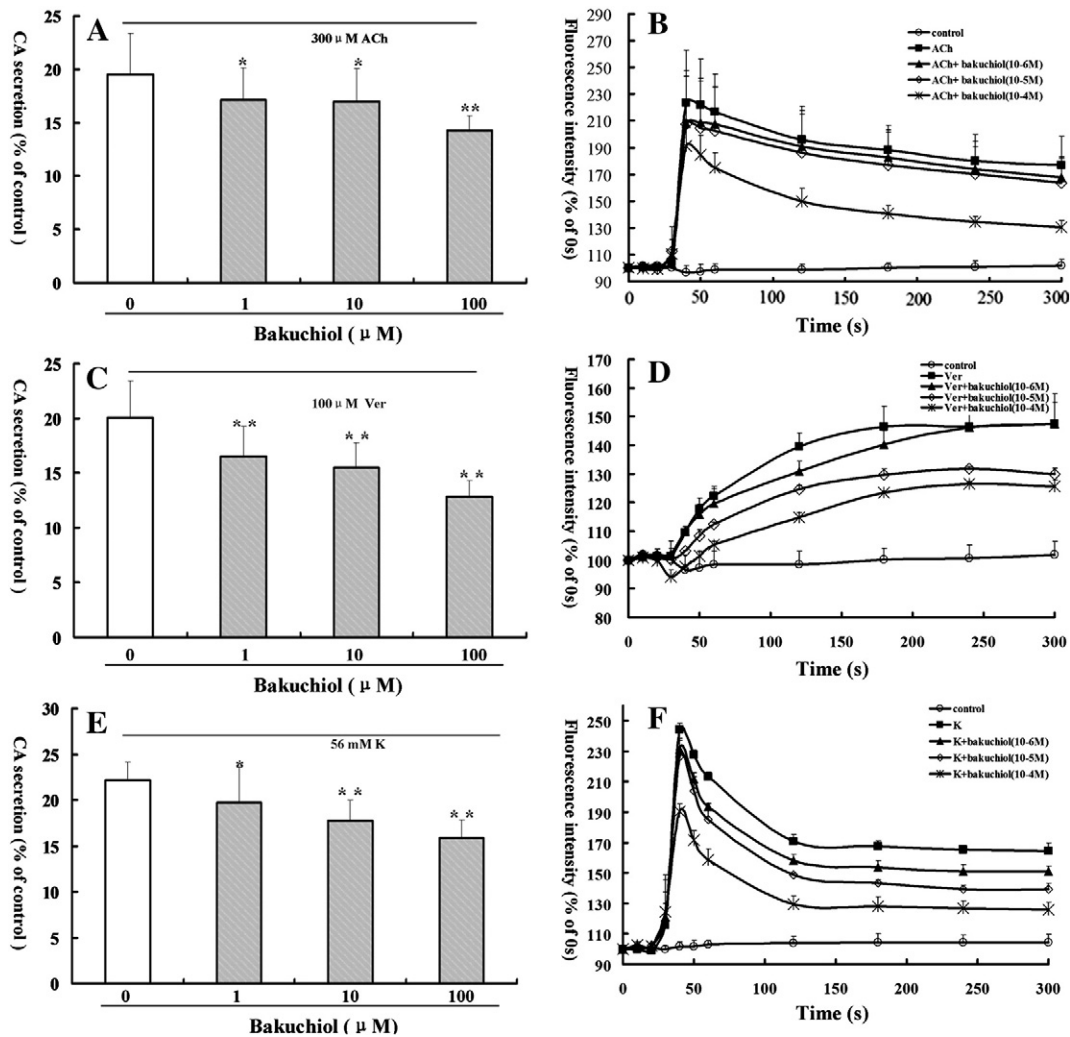
TH Ser31 phosphorylation induced by 100 μM bakuchiol was time sensitive. The level of TH Ser31 phosphorylation peaked at 30 min

(*p* < 0.01, Fig. 6A), subsequently decreased after 60 min. 100 μM bakuchiol significantly stimulated catecholamine secretion (vs. baseline, *p* < 0.01, Fig. 6B), while incubation with 100 μM bakuchiol and 10 μM PD98059, an antagonist of the ERK pathway, for 30 min, significantly attenuated phosphorylation of TH Ser31 (vs. bakuchiol, *p* < 0.01).

TH Ser40 phosphorylation was also promoted and peaked at 30 min (*p* < 0.01, Fig. 6C) by 100 μM bakuchiol which also significantly stimulated CA secretion (vs. baseline, *p* < 0.01, Fig. 6D). H89 (10 μM), a PKA pathway antagonist, significantly reduced phosphorylation of TH Ser40 (*p* < 0.01) induced by 100 μM bakuchiol.

*Inhibition of bakuchiol on CA secretion and the elevation of [Ca<sup>2+</sup>]<sub>i</sub> induced by various secretagogues in bovine adrenal medullary cells*

In order to characterize the inhibition of bakuchiol on nAChR-ion channels, voltage-dependent Na<sup>+</sup> channels, and voltage-dependent Ca<sup>2+</sup> channels, we studied the effects of 1–100 μM bakuchiol on CA secretion and increase [Ca<sup>2+</sup>]<sub>i</sub> induced by ACh, Ver and 56 mM K<sup>+</sup>. Incubation of bakuchiol (1–100 μM) for 10 min significantly reduced ACh (*p* < 0.01, Fig. 7A), Ver (*p* < 0.05 or 0.01, Fig. 7C) and 56 mM K<sup>+</sup> (*p* < 0.05 or 0.01, Fig. 7E) induced secretion compared to various secretagogues alone. By detecting the intracellular calcium concentration, we found that bakuchiol (1–100 μM) suppressed [Ca<sup>2+</sup>]<sub>i</sub> increase induced by ACh (Fig. 7B), Ver (Fig. 7D) and 56 mM K<sup>+</sup> (Fig. 7F) in a concentration dependent manner, which coincides with the results of CA secretion.



**Fig. 7.** Inhibition of bakuchiol in CA secretion and the elevation of  $[Ca^{2+}]_i$  induced by various secretagogues on bovine adrenal medullary cells. Effects of bakuchiol on ACh-induced CA secretion (A) and  $[Ca^{2+}]_i$  (B). Effects of bakuchiol on Ver-induced CA secretion (C) and  $[Ca^{2+}]_i$  (D). Effects of bakuchiol on 56 mM  $K^+$ -induced CA secretion (E) and  $[Ca^{2+}]_i$  (F) ( $n = 8$  or 9 per group). \* $p < 0.05$ , \*\* $p < 0.01$ .

#### Effects of bakuchiol against acute stress by FST and TST

We demonstrated in the open field test that bakuchiol at the dose of 15 mg/kg to 60 mg/kg did not affect the numbers of crossing ( $p > 0.05$ , Fig. 8A) and rearing ( $p > 0.05$ , Fig. 8B). Results of TST show that 10 mg/kg venlafaxine, and 30 mg/kg and 60 mg/kg bakuchiol significantly declined immobility time compared with the vehicle group ( $p < 0.01$  or 0.05, Fig. 8C). FST results showed that 10 mg/kg venlafaxine and 30 mg/kg bakuchiol significantly declined immobility time compared with the vehicle group ( $p < 0.01$  or 0.05, Fig. 8D).

#### Effects of bakuchiol on chronic stress

Seven-day stress induced a significant increase of plasma norepinephrine ( $p < 0.05$ , Fig. 9A) and epinephrine ( $p < 0.05$ , Fig. 9B) compared to the control group. Treatments with 30 mg/kg and 60 mg/kg bakuchiol significantly attenuated plasma norepinephrine induced by stress ( $p < 0.05$ ). Bakuchiol at 60 mg/kg significantly attenuated plasma epinephrine induced by stress on the 7th day ( $p < 0.05$ ).

#### Discussion

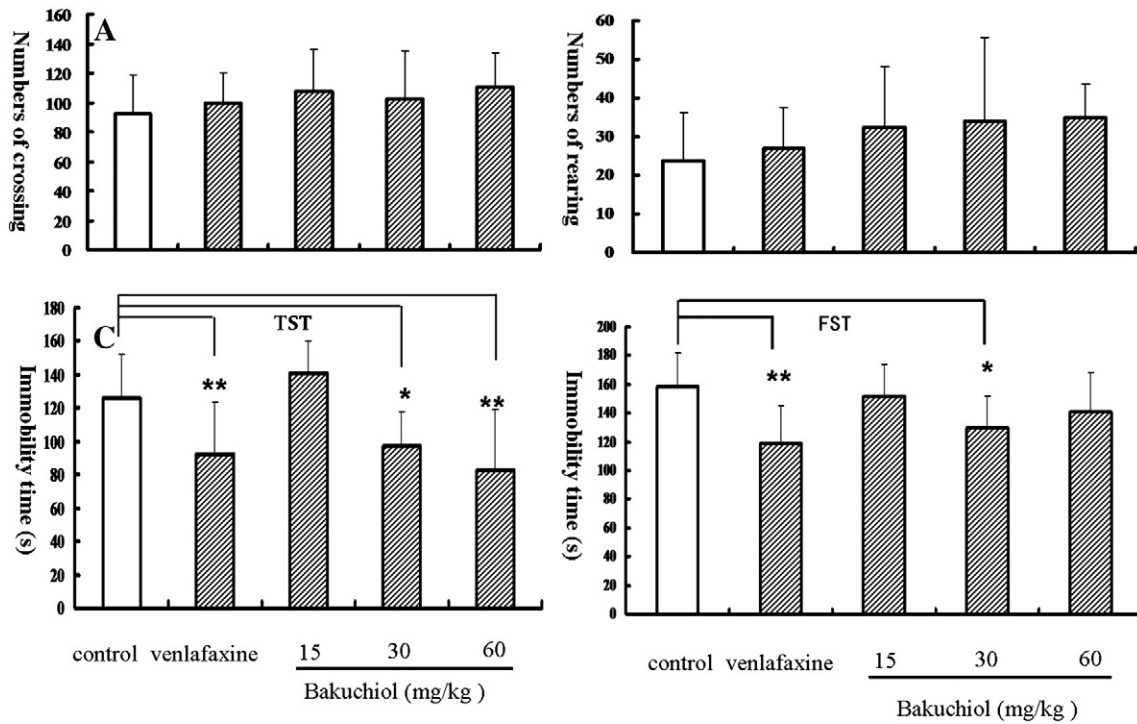
In the present study, we demonstrated that bakuchiol had a dual effect on CA secretion in cultured bovine adrenal medullary cells. It inhibited CA secretion induced by various secretagogues, while it

significantly stimulated the basal CA secretion when it was used alone (Fig. 2). To the best of our knowledge, this is the first direct evidence showing the dual effects of bakuchiol on CA secretion.

Although, the exact mechanisms are still unknown, the rise in cellular  $Ca^{2+}$  concentration is a prerequisite for the synthesis and secretion of CA.  $Ca^{2+}$  concentration in the cells is affected by  $Ca^{2+}$  channels in the cell membrane and/or intracellular calcium pools (Stauderman et al., 1990). 10  $\mu$ M nitrendipine, a  $Ca^{2+}$  channel blocker, decreased 100  $\mu$ M bakuchiol-induced CA secretion, indicating that the effect is mediated by activating the membrane  $Ca^{2+}$  channels in the cell membrane. 100  $\mu$ M bakuchiol significantly stimulating the basal CA secretion in calcium free medium means that bakuchiol also recruit  $Ca^{2+}$  from calcium pools inside of the cells to stimulate CA secretion.

Activation of MAPK pathway is involved in stress-induced CA secretion and synthesis (Cortez et al., 2012; Sekine et al., 2011; Wang et al., 2010). ERK1/2, SAPK/JNK, p38 MAPK are members of the MAPK family. Bakuchiol promotes the phosphorylation of ERK1/2 and SAPK/JNK pathways but had no effect on the phosphorylation of p38 MAPK pathway. Interestingly, only SAPK/JNK pathway antagonist, SP600125, inhibited bakuchiol induced CA secretion. Instead of inhibiting CA secretion, the ERK1/2 pathway antagonist U0126 further stimulated the CA secretion induced by bakuchiol. We got the same results by using another antagonist of ERK1/2 pathway PD98059 (data not shown). Although more detailed mechanisms remain to be revealed, our work shows that selective activation of SAPK/JNK pathway by bakuchiol is at least in part



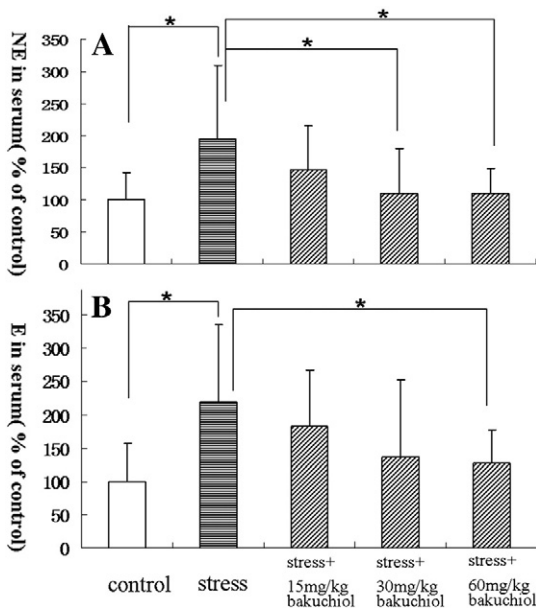


**Fig. 8.** Anti-depressant like effects of bakuchiol in mouse. (A) Effect of bakuchiol on crossing (A) and rearing (B) ( $n = 8-10$  per group) in open field test. (C) Effect of bakuchiol on the immobility in TST ( $n = 9$  or  $10$  per group). (D) Effect of bakuchiol on the immobility in FST ( $n = 9$  or  $10$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ , compared with control.

involved in the stimulation of CA secretion from bovine adrenal medullary cells.

Our fluorescence polarization assay of receptor binding showed that bakuchiol has a strong affinity for ER, especially for ER $\beta$ . Despite the discrepancy on the selective affinity for ER subfamilies between our and Lim et al.'s (2011) results, both works confirmed that bakuchiol had a phytoestrogen-like effect as we have reported previously that bakuchiol had strongly activated transcription of both ER $\alpha$  and ER $\beta$ , especially on ER $\beta$  (Xin et al., 2010). It coincided with the binding affinities of bakuchiol showed in the present work.

Activation of TH, a rate-limiting enzyme in CA synthesis, by phosphorylation is the primary mechanism responsible for the maintenance of CA levels in tissues. The activity of TH can be regulated by phosphorylation at several serine (Ser) residues: Ser 8, Ser19, Ser31, and Ser40 in the NH $_2$ -terminal domain. The phosphorylation of TH at Ser40 and Ser 31, but not at Ser19 or Ser8, can directly increase TH activity. However, phosphorylation of TH at Ser31 increases the activity of TH to a much lesser extent than that at Ser40. Phosphorylation at Ser19 increases the rate of Ser40 phosphorylation leading to an increase in enzyme activity (Dunkley et al., 2004). Our results suggest that bakuchiol might increase CA secretion by stimulating CA synthesis through the activation of TH at sites Ser31 and Ser40.



**Fig. 9.** Role of bakuchiol against chronic immobilization stress in mouse. (A) Effect of bakuchiol on NE secretion in serum ( $n = 7-9$  per group). (B) Effect of bakuchiol on E secretion in serum ( $n = 7-9$  per group). \* $p < 0.05$ .

Recently, accumulated studies reported that estrogen and phytoestrogen regulated the CA system by various manners and mechanisms. Lopez et al. (1991) reported that 17 $\alpha$ -estradiol inhibited CA secretion induced by DMPP, methacholine and 120 mM K $^+$ . The Ca $^{2+}$  uptake into cells in cultures stimulated by DMPP or high K $^+$  was almost inhibited completely by 100  $\mu$ M 17 $\alpha$ -estradiol. Yanagihara et al. (2006) reported that 17 $\beta$ -estradiol (E $_2$ ) (0.3–100 nM) stimulated CA synthesis from tyrosine by the activation of tyrosine hydroxylase. The same results were seen in phytoestrogens such as daidzein (0.01–1.0  $\mu$ M), a soy isoflavone, and resveratrol (0.1–1.0  $\mu$ M), a grape polyphenol which stimulated CA synthesis (Liu et al., 2007; Shinohara et al., 2007). However, 17 $\beta$ -estradiol (E $_2$ ) (Kim et al., 2000), daidzein and resveratrol at high concentrations ( $\geq 1.0$   $\mu$ M) inhibited catecholamine secretion induced by various secretagogues such as ACh, Ver and 56 mM K $^+$ . Another phytoestrogen cnicifugoside did not affect the KCl-induced secretion but markedly inhibited the DMPP-induced CA secretion in bovine chromaffin cells (Woo et al., 2004). Nonylphenol (NP) is a compound with estrogenic activities and has showed a similar effect on CA secretion like bakuchiol. NP alone elevated the basal secretion of CA in a dose-dependent manner and NP partially suppressed DMPP induced CA release (Liu et al., 2008). Butyl benzyl phthalate (BBP), a plasticizer and an environmental pollutant, exerts genomic estrogenic-like effects via estrogen receptors. It inhibited the DMPP-induced secretion of CA in a dose-dependent manner (IC $_{50}$  measured as 5 and 6 mM, respectively) (Liu et al., 2003). We firstly found that



stimulation of bakuchiol on basal CA secretion may be related with its phytoestrogenic-like effect.

Although CAs are known to be neurotransmitters in the regulation of normal functions in healthy humans, CA deficiency can also lead to various diseases such as mental depression (Getachew et al., 2010), memory loss (Gibbs et al., 2010), Parkinson's disease (Kita and Kita, 2011) and Alzheimer disease (Liu et al., 2011; Yu et al., 2011). In these diseases, depression is also related with the changes of estrogen levels. Ovariectomized rats encountered an augmented amount of depressive behavior (Al-Rahbi et al., 2013). Women were at a higher risk than men to develop mood disorders and depression. The increased risk is associated with fluctuating estrogen levels that occur during menopausal transition, a time characterized by drastic fluctuations in estrogen levels (Wharton et al., 2012). Based on the in vitro findings, we hypothesized that phytoestrogen bakuchiol might be beneficial in the treatment of depression. So TST and FST were employed to check our hypothesis. Venlafaxine, selected as the positive control in the present study, is one of the most widely used clinical antidepressant. Compared with the control group, 30 mg/kg and 60 mg/kg bakuchiol reduced the immobility time in the TST in a concentration dependent manner ( $p < 0.01$  or  $0.05$ ), and 30 mg/kg bakuchiol also significantly reduced the immobility time in the FST ( $p < 0.05$ ). Although it is not significant, 60 mg/kg bakuchiol also showed a tendency which can reduce the immobility time. These suggest that bakuchiol may possess the potential antidepressant like effect but further experiments of bakuchiol should not be more than 60 mg/kg. Since TST and FST were two simple tests for screening, the antidepressant like effect of bakuchiol and the exact mechanisms remain to be studied. There were no differences in crossings and rearing between the control group and bakuchiol treated groups in the open field test (OFT). It means that there is no association of the immobility time in the FST and TST with the alterations in locomotor activity. FST and TST results are consistent with the findings in vitro and suggest that bakuchiol might be a potential candidate for the treatment of depression.

It was reported that antidepressant medication with estrogen replacement therapy (ERT) may speed the onset or augment the response of antidepressant action (Rasgon et al., 2007). In a recent study, the influence of the ERT on the antidepressant response to sertraline (50–150mg) in 127 women over 60 years old was evaluated. It found that women receiving ERT had significantly greater improvement and quality of life than those not receiving ERT (Schneider et al., 2011). Our work suggested that bakuchiol as a phytoestrogen may augment the antidepressive effects of classical selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepinephrine reuptake inhibitors (SNRIs) when co-administrated. Further studies are required to prove this possibility.

As a kind of neurotransmitter in the regulation of normal functions in healthy humans, deficiency of CA can be harmful. However, over excitation of the CA system caused by long-term stress is also thought to contribute to the involvement and the exacerbation of hypertension, coronary heart disease, heart failure (Esler and Kaye, 2000; Esler et al., 2008), gastric ulcers (Veenstra et al., 2007) and diabetes (Peschke et al., 2011), meanwhile it can promote aging, tumorigenesis, neuropsychiatric conditions and miscarriages by DNA damage (Hara et al., 2011). Our experiments by using a daily immobilization stress model showed that phytoestrogen bakuchiol significantly reduced stress induced raise of serum CA level. Recently, more and more experimental data and epidemiological studies reported that estrogen may play an important role in avoiding cardiovascular diseases (Maulik et al., 2012; Pedram et al., 2013). Our result was consistent with the report that the protection of cardiovascular system by estrogen may be related to the reduction of CA overflow (Fukumoto et al., 2012). The results are consistent with the findings in bovine adrenal medullary cells and suggest that bakuchiol might be beneficial for the treatment of stress.

In summary, the present study established that bakuchiol stimulated basal CA secretion by the JNK pathway through its phytoestrogenic activity, while inhibiting CA secretion induced by various secretagogues.

This alluded to the fact that bakuchiol may exert different effects on CA secretion. The study also gives some hints for the potential clinical use of bakuchiol in the treatment of depression and stress related disorders.

### Conflict of interest

The authors declare that there are no conflicts of interest.

### Acknowledgments

We thank Abudulai Jaw Bah and Oche Barnabas for proofreading this manuscript. This study was supported by the National Natural Science Funds (81125024; 81001660; 30830121; 81303247), the National Basic Research Program of China (2012CB518404), the National Science and Technology Major Project of China "Key New Drug Creation and Manufacturing Program" (2012ZX09304007) and the National Scientific and Technological Pillar program (2012BAI05B01), Research Fund for the Doctoral Program of Higher Education of China (20101210110002, 20121210120009).

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