Evidence Based for Improved Pharmacological Efficacy and Decreased Toxicity by Traditional Processing of Rhizoma Arisaematis in Mice

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Abstract:

Rhizoma Arisaematis (RA, the rhizome of *Pinellia pedatisecta* Schott) is a Traditional Chinese medicine commonly used for the management of convulsion, inflammation, and cancer. Despite being used for more than two thousand years, its pharmacological and toxicological effects by traditional processing products are still unclear. In this study, we attempted to investigate the neurobiological effects exerted by the different preparations of RA treated with either Alumen plus ginger juice (Zhinanxing) or with bile juice (Dannanxing) as compared with those of the untreated crude RA in ICR mice. The results showed that both the Zhinanxing and Dannanxing extracts exerted significantly more sedative effects as revealed by the inhibitory effects on ambulatory distances, jump, vertical-plan entries and prolonged pentobarbital-induced sleeping time as well as the analgesic property (increase of tail flick latency in the nociceptive testing) on mice than the unprocessed crude RA after oral administration for 1- to 3-days and after 18 days cessation of treatment. By contrast, the toxic effects such as the increase of stereotype-1 episodes of locomotor activities and reduction of the retention time on a rotating rod (motor equilibrium dysfunction) were found in mice only treated with unprocessed crude RA for 3 consecutive days, which were persistent after 18 days of cessation of administration. These neurotoxic effects were accompanied with an increase in plasma lipid peroxidation (LPO), decrease of whole blood nitric oxide (NO_x) levels, and inhibition of Na^+/K^+-ATPase activities on the membrane fractions of erythrocytes

and cerebral cortex. In conclusion, these findings provide scientific evidence that the processed RA indeed possessed not only enhanced pharmacological efficacies but also reduced noxious effects as compared with the unprocessed crude RA. The signaling of NO/oxidative stress/Na^+-K^+-ATPase played a role, at least in part, in the underlying mechanisms of neurotoxic effects induced by the crude RA.

Keyword: Rhizoma Arisaematis; Traditional Chinese medicine; Sedation; Analgesia; Neurotoxicity; Na^+/K^+-ATPase activities.

Introduction:

Rhizoma Arisaematis (RA), the rhizome of *Pinellia pedatisecta* Schott, is characterized with bitter, warm, pungent, and toxic properties (Sun *et al.*, 1995). It is a traditional Chinese medicine (TCM) and has long been used as sedatives, stomachic, analgesic, anti-coagulants, anti-inflammatory, anti-emetic, and anti-tumor (Mao *et al.*, 2002 and 1994; Zhu *et al.*, 1999). In TCM books, the possess efficacy of RA in dispelling wind and relieving convulsion, eliminating stagnation, and drying dampness to eliminate phlegm has also recorded. Chemical analysis of the probably active components of RA include beta-sitosterol, total alkaloids, guanosine, γ-aminobutyric acid and dipeptides (Qin *et al.*, 1984; Wang *et al.*, 1997; Wu *et al.*, 1995). Thus, to date, it still remains indispensable for the management of vertigo, epilepsy, tetanus, stroke, hemiparalysis, fever, and cancer. However, the crude RA also exhibited toxic effects especially an improper dosage used could lead to serious toxicities including mucous membrane inflammation and necrosis, parageusia, paralysis of motor nerve terminals and central motor area, convulsion, asphyxia, irregular respiration, and eventually death due to respiratory paralysis (Sun *et al.*, 1993; Tomlinson *et al.*, 2000). In addition, the crude RA also caused mental retardation in children and verbal learning impairment.

It has long been known that the crude RA is very toxic and must be processed carefully. According to the art of traditional Chinese processing from Pharmacopoeia, the crude RA should be processed with either an optimum amount of Alumen (Aluminum Potassium Sulfate,

$KAl(SO_4)_2 \cdot 12H_2O$) plus ginger juice (an alias: Zhinanxing) or bile juice (an alias: Dannanxing) (He *et al.*, 1997; Wu *et al.*, 1995 and 1996). It was claimed that these different processes, not only enhance the pharmacological efficacies, but also attenuate the toxic side effects of the crude RA (Wu *et al.*, 1998). Although the differently processed products of RA have been used in the clinical practice in the traditional Chinese medicine, it still needs experimental evidence to delineate the neuropharmacological properties, toxicological profiles, and their possible action mechanisms.

In this study, we attempted to explore the neuropharmacological and toxicological effects exerted by the differently processed Rhizoma Arisaematis (RA), namely Zhinanxing and Dannanxing, as compared with the crude RA in mice. We also investigated the effects of these water extract (1 g/kg/day for 3 consecutive days) on various neuro-behaviors (spontaneous locomotor activities, prolonged pentobarbital-induced sleeping time, tail flick reflex testing, and motor equilibrium performance) as well as the biochemical effects on the blood and brain tissues. The expected results would provide evidence to support that the processed RA products (with either Alumen plus ginger juice or bile juice) may not only enhance the pharmacological efficacies but also reduce the toxic effects of the crude RA.

Materials and Methods:

Materials

The crude Rhizoma Arisaematis (RA, the rhizome of *Pinellia pedatisecta* Schott) was obtained and ensured the authentication of this medicinal plant and quality from Committee on Chinese Pharmacy, Department of Health, Executive Yuan, Taipei, Taiwan. As stipulated in the pharmacopoeia, the processed technology of RA, with the dry rhizome of *Pinellia pedatisecta* Schott, was obtained after treatment with Alumen (Aluminum Potassium Sulfate, $KAl(SO_4)_2 \cdot 12H_2O$) plus ginger juice (Zhinanxing), or with bovine bile juice (Dannanxing) (He *et al.*, 1997; Wu *et al.*, 1997). The water extracts of the different processed samples (including: Zhinanxing, Dannanxing, and the crude RA) were prepared by macerating the Rhizoma Arisaematis with hot water (1g of dry sample in 1 ml of 95℃ distilled water) and then cooled at room temperature. The water extracts (supernatants) were obtained by centrifugation at 12,000 g x 10 min. The drug concentration of the extract was thus regarded as 1g per 1 ml of the supernatant.

Experimental design

Randomly bred adult male ICR mice (age 4-6 weeks) weighing 18-22g were obtained from the Animal Center of the College of Medicine, National Taiwan University (Taipei, Taiwan). The care and use of laboratory animals were conducted in accordance with the guidelines

from the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University. Mice were housed six per cage under standard laboratory conditions at an ambient temperature of 22.1 ± 1.5 ℃ and moisture of 40～60%. The mice were given a solid diet and tap water ad-libitum and 12hrs for daily and night respectively. The animals were acclimatized to the laboratory conditions prior to the experiments and all experiments were carried out between 10:00 AM and 05:00 PM.

The water extracts of the different processed RA samples were administered to the mice (1 g/kg/day) once daily by oral gavage for 3 consecutive days (eight mice per each group) and the following parameters were measured: body weight, spontaneous locomotor activities, prolonged pentobarbital-induced sleeping time, nociceptive testing (tail flick reflex), and motor equilibrium performance tested on the rotating rotarod treadmill (60rpm). Nitric oxide (NO_x) concentrations in the whole blood were collected daily after administration of the water extracts and on 4, 11, and 18 days after cessation of administration. Moreover, we assessed the plasma lipid peroxidation levels, and Na^+/K^+-, Mg^{2+}-ATPase activities of the erythrocyte membrane after administration of the three different processed Rhizoma Arisaematis water extracts for 3 consecutive days, and/or after 4 days cessation of administration, respectively.

Spontaneous locomotor activity

The spontaneous locomotor activity tests were performed during the day (9:00-18:00). When the drugs were administered by consecutive oral route, the mice were individually placed in an open field. A large colorless rectangular box with a metallic grid floor was used (70-cm wide, 90-cm long and 60-cm high). The photobeam activity monitors (Tuscan Coulbourn instruments) and real-time for detecting track-type plots were used. Overall pulses were recorded in an electromechanical counter as a gross measure of activity. Typical application of X-Y activity recording (floor plane activity) sensory ring drops over the cage and rests on ring support. Movement was detected by 16 × 16 infrared photobeam detectors and transducers set 1.5 cm above the floor of the apparatus and measured by a PC. Finally, the number of squares crossed and the plots of tracking were counted during a period of 30 min for all experiments and quantification of data was by TruScan99 software. The mice were orally administered with the water extracts of different processed Rhizoma Arisaematis or distilled water (vehicle control) once every day for 3 consecutive days and the effects on the spontaneous locomotor activity were measured.

Pentobarbital-induced sleeping time

The mice were orally administered with the water extracts of different processed RA (1 g/kg/day) or distilled water (vehicle control) once every day for 3 consecutive days. The prolongation of sleeping time was induced by an intraperitoneal injection of pentobarbital (50

mg/kg) and recorded the sleeping time from loss to recovery of righting reflex on 3 days of drugs administration and after 4, 11, and 18 days cessation of administration, respectively (Huang *et al.*, 2007).

The nociceptive testing (tail flick reflex)

Pain thresholds were assessed by means of a modification of tail flick (TF) test (Hara *et al.*, 1998). All TF testings occurred in a dimly lit room maintained at 27℃. The mice were loosely held in plexiglas cyclinders, constructed so that each mouse tail protruded from the rear of cyclinder. This allowed TF testing to occur without disturbing the animals. Following a 30-min habituation period, baseline and TF latencies were recorded. The tail was placed over a radiant heat source (50 W projector lamp) adjusted to provide baseline latencies in naive animals of approximately 10s. A cut-off latency of 30s was used to avoid tissue damage to the tail. The mice were orally administered with the water extracts of different processed Rhizoma Arisaematis or distilled water (vehicle control) once every day for 3 consecutive days and the effects on tail flick (TF) test were measured at 1, 2, and 3 days of drugs administration and after 4, 11, and 18 days cessation of administration, respectively.

Motor equilibrium performance

The effect of the different processed RA on motor coordination in the separate groups of the

mice was tested using an accelerating rotating rod treadmill (Ugo Basile; Stoelting Co., Chicago, IL), as described previously (Huang *et al.*, 2007). The rotating rod was set in motion at a constant speed (60 rpm) and the mice were placed on individual sections of rotating rod. Each time an animal fell, it was noted whether the fall had occurred when it sat still or when it walked. Three successive trials separated by a 10 minutes pause were performed. The mice were orally administered with the water extracts of different processed Rhizoma Arisaematis or distilled water (vehicle control) once every day for 3 consecutive days and the effect on motor equilibrium performance was measured.

Measurement of nitric oxide (NO_x) production

The quantitative nitric oxide (NO_x) assay was based on that described previously (Huang *et al.*, 2008). Briefly, the whole blood sample of the mice was collected to eppendorf from an eyehole vessel before treatment with drug (day 0) after light anesthesia by an intraperitoneal injection of pentobarbital (25 mg/kg). Then the mice which were orally administered with the water extract of crude RA once every day for 3 consecutive days were collected the whole blood sample at 1 and 3 days of drugs administration and after 4, 11, and 18 days cessation of administration, respectively. To avoid total protein denatured incompletely, we added 95% ethanol into the blood in the eppendorf at 4℃ overnight (12-16 hrs). Next day, all samples were centrifuged at 4℃ for 20 min at 12000 × g. The supernatants of these samples were

collected and assayed by the NO/ozone chemiluminescence (NO Analyzer 280A SIEVERS) for quantitative NO_x levels which measured the oxidation products (NO_2- and NO_3-) of NO using a reaction vessel containing a reducing system (0.1 M vanadium chloride, Aldrich Co., Germany). Detection of NO_x is then completed by its reaction with ozone, which leads to the emission of red light ($NO + O_3 \rightarrow NO_2^* + O_2$; $NO_2^* \rightarrow NO_2 + h\nu$). Standard curves were made prior to concentration (1, 5, 10 15 and 20 μM NO), which were prepared using freshly prepared solutions of $NaNO_2$ in distilled water.

Plasma lipid peroxidation (LPO) assay

The method of formation of malondialdehyde (MDA), a substance produced during lipid peroxidation, was assayed as described previously (Huang *et al.*, 2007). The assay is based on the reaction of a chromogenic agent, N-methyl-2-phenylindole, with MDA to form an intensive colored carbocyanine dye. The optical density of the color was detected by an enzyme-linked immunosorbent assay (ELISA) microplate reader and absorption was 586 nm. The plasma samples were added 3.25 volumes of diluted R1 reagent (10.3 mM *N*-methyl-2-phenylindole in acetonitrile), followed by gentle vortex mixing. After addition of 0.75 volumes of 37 % HCl, the mixtures were incubated at 45°C for 60 min. After cooling, the absorbance of the clear supernatant was read at 586 nm. The linearity of the standard curve was confirmed with 0, 5, 10, 20 and 40 μM MDA standard (1, 1, 3,

3-Tetramethoxypropane). The protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Na^+/K^+-ATPase activity assay

Membrane Na^+/K^+-ATPase activities were assayed as described previously (Huang *et al.*, 2008). The method allowed for quantification of two distinct Na^+/K^+-ATPase and Mg^{2+}-ATPase activities in the same sample. The enzymatic activities were measured in triplicate in covered 96 well microliter plates at 37 ± 0.5 ℃ on a shaker. Thirty microliters of assay buffer (118 mM NaCl, 1.67 mM KCl, 1.2 mM $MgCl_2$, 12.3 mM $NaHCO_3$, 11 mM glucose, 0.5 mM EGTA, PH:7.4) containing 2 μg of membrance protein was added to each well. The Na^+/K^+-ATPase activity were determined by subtracting the ouabain (1.25 mM) insensitive Mg^{2+}-ATPase activity from the overall $Na^+/K^+/Mg^{2+}$-ATPase and the assay was started with the addition of 10 μl of ATP (final concentration 5 mM) making the final reaction volume of 100 μl. The reaction was terminated after pre-incubation at 37 ± 0.5 ℃ by the addition of 200 μl of malachite green (MG) plus ammonium molybdate (AM) (3:1). The inorganic phosphate (Pi) released from the substrate ATP was colorimetrically assayed by a microplate ELISA reader (Dynatech MR7000, Ashford, Middesex, UK) at 630 nm. The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that included in the assay procedure. The

specific ATPase activities were expressed as Pi μmole (micromoles inorganic phosphate) released per mg protein per hr. Values reported represent mean ± S.E. of at least three separate experiments.

Statistical analysis

The results in the text are given as mean ± standard errors (S.E.). The significance of difference was evaluated by the Student's *t* test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA); the Duncan's post-hoc test was applied to identify group differences. The *P* value less than 0.05 was considered to be significant. The statistical package SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

Results:

Effect of different Rhizoma Arisaematis (RA) processes on locomotor activities and sleeping times

The sedative effects of drugs were quantitatively measured by decreased spontaneous locomotor activities and prolonged sleeping time (Huang *et al.*, 2007). The mice, which equally divided into three groups, were orally administered (1g/kg/day) with one of the the three kinds of Rhizoma Arisaematis (RA) preparations, either processed by alumen plus ginger juice (Zhinanxing), by bile juice (Dannanxing), or crude R.A., respectively for 3 consecutive days. As shown in Fig. 1, mice treated with Dannanxing and R.A. for 2 days exhibited a significantly hypoactive effect in the quantitative ambulatory distances, while those treated with Zhinanxing, a decreased effect was only observed after administration for 3 consecutive days. This effect was sustained even for 18 days after cessation of administration in the Dannanxing and crude R.A. group (Fig. 1A).The frequencies of jump and vertical-plane entries were also suppressed in a similar order of potency as decreased ambulatory distance (Figs. 1B and 1D). However, the frequency of stereotype-1 episodes was slightly increased only in the group administrated with crude RA group for day 2 and day 3 ($^{*}p < 0.05$ as compared with vehicle control group), and the effect was sustained for 18 days even after cessation of administration (Fig. 1C).

To further explore the effect of RA extracts-induced sedation, we analyzed the RA

extracts-caused prolongation of pentobarbital-induced sleeping time. Oral administration of these three kinds of RA extracts (1 g/kg/day) for 3 consecutive days exerted a marked prolongation of sleeping time induced by pentobarbital (increased by 199.4 ± 16.4 % (Zhinanxing), by 252.5 ± 13.6 % (Dannanxing), and by 175.4 ± 14.4 % (crude RA) as compared with control group, $^{*}p < 0.05$, Fig. 2); Dannanxing extract significantly exhibited higher potency than Zhinanxing and crude R.A. extracts ($^{\&}p < 0.05$ as compared with Zhinanxing group, and $^{\#}p < 0.05$ as compared with crude RA group). This effect of Zhinanxing as well as Dannanxing, but not that of crude RA sustained after 4 days cessation of administration, and then gradually restored normally after 18 days of cessation.

Effect of different RA *processes on antinociceptive measurementt*

To understand whether different RA processes could affect the nociceptive responses, we investigated the analgesic effects of the three kinds of RA extracts in mice by analyzing the pain thresholds using a modified tail flick reflex test. As shown in Fig. 3, it was found that there was a definite inhibitory effect on the thermal induced hyperalgesia (analgesic effect) after administration with Zhinanxing and Dannanxing extracts, but not crude RA extract, for 2 and 3 consecutive days. The analgesic effect of Zhinanxing and Dannanxing was still persistent even after 4 days cessation of administration, and all of these effects restored normally at 11 days after cessation of administration.

Effect of different RA processes on motor equilibrium performance dysfunction on a rotating rod

It has been reported that the motor equilibrium performance on a rotating rod is a more complex motor skill task, which required both fine motor coordination and precise postural control, and thus useful for monitoring the neurotoxic effect of poisons or drugs (Huang *et al.*, 2007). To examine the RA-induced neurotoxicity after exposure to different extracts, the motor equilibrium performance was determined by measuring the retention time on a rotating rod (60 rpm). The retention time performed in mice administrated with the crude RA extract (1g/kg/day) was significantly decreased on day 1 and the extent of this effect was persistent even after 18 days cessation of administration. However, neither Zhinanxing nor Dannanxing induced motor equilibrium dysfunction (Fig. 4).

Crude RA induced the inhibition of nitric oxide (NO_x) levels in the whole blood

NO_x is an important signaling molecule (a diffusible multifunctional second messenger) that mediates several physiological functions, including the regulation of neurotransmission (Moncada and Higgs, 1991). However, the important role of NO_x in the crude RA-induced toxicological effects remained unclear. The aim of this investigation was to ascertain whether crude RA induces the neurotoxicity via effect on whole blood nitric oxide (NO_x) (alteration of

locomotor activities and motor equilibrium performance dysfunction). As shown in Figure 5, the whole blood NO_x levels was significantly decreased (83.3 ± 4.9 % as compared with control: 100 ± 7.1 %) after administration with the 1 g/kg/day crude RA extract for 3 consecutive days, and this effect persistently declined even after 18 days cessation of administration with crude RA (after 4 days (70.6 ± 4.5 %), 11 days (66.4 ± 4.7 %), and 18 days (80.5 ± 3.2 %) as compared with control group, respectively, $^{*}p < 0.05$).

Crude RA triggered the increase of lipid peroxidation (LPO) production and inhibition of Na^+/K^+- and Mg^{2+}-ATPase activities

To investigate the possible mechanism of the crude RA-induced neurotoxic effects, we treated the mice with crude RA extract and analyzed plasma LPO production as an indicator for reactive oxygen species (ROS) formation and influence of Na^+/K^+- and Mg^{2+}-ATPase activities (the most sensitive biomarker of ROS (24)) in whole blood. As shown in Fig. 6, significantly increased LPO levels were observed in plasma after administration with 0.33 and 1 g/kg/day of crude RA extract for 3 consecutive days, which sustained after 4 days cessation of administration. Meanwhile, Na^+/K^+- and Mg^{2+}-ATPase activities of erythrocyte membranes were also remarkably inhibited after 4 days cessation of administration, (Fig. 7A).

Furthermore, the crude RA extract, but not Dannanxing extract, significantly inhibited Na^+/K^+-ATPase activities in isolated cerebral cortex in a concentration-dependent manner

ranging from 0.125 to 1 mg/ml (inhibited to 98.3 ± 5.7 %, 80.9 ± 3.2 %, 71.0 ± 6.1 %, and 68.5 ± 5.0 % by the crude RA extract 0.167, 0.33, 0.5, and 1 mg/ml respectively, as compared with control group, $^{*}p < 0.05$, Fig. 7B). Therefore, it is implied that the crude RA-induced neurotoxicity may be correlated with the ROS formation and inhibition of Na^{+}/K^{+}-ATPase activities.

Discussion:

Rhizoma Arisaematis (RA, the rhizome of *Pinellia pedatisecta* Schott) extract has been used in the traditional Chinese medicine for thousands of years mostly as anticonvulsant, analgesics, sedative, stomachic, expectorant, anti-coagulant, anti-inflammation, anti-tumor and remission of dysmenorrhea (Li *et al.*, 2004 and 2010; Zhu *et al.,* 1999). However, the crude RA has toxic properties and when used inappropriatly could induce serious toxic effects. Some studies have indicated that the processing technologies of crude RA by either with an optimum amount of Alumen (Aluminum Potassium Sulfate, $KAl(SO_4)_2 \cdot 12H_2O$) plus ginger juice (an alias: Zhinanxing) or with bile juice (an alias: Dannanxing) can enhance the effective component contents and decrease the toxic effects (Wu *et al.*, 1995 and 1999). Despite description on the pharmacological and toxicological effects of RA has been reported, the precise mechanism of action of either crude or processed (Zhinanxing or Dannanxing) RA still remain unclear. To investigate the efficacy of differently processed RA (Zhinanxing, Dannanxing or crude RA), we first investigated the sedative and analgesic effects of the three kinds of RA extracts in ICR mice. Our results revealed that: (1) the suppressive parameters of locomotor activities including ambulatory distances, jump frequencies and vertical-plane entries were induced after administration with 1 g/kg/day of the three kinds of RA extracts for 3 consecutive days and the effects were sustained for 18 days after cessation of administration (Fig. 1); (2) all of three extracts significantly prolonged the sleeping time induced by

pentobarbital after oral administration, but the potency was observed higher in that of the Dannanxing group than those of Zhinanxing and crude RA group. Four days after the cessation of administration, Dannanxing and Zhinanxing, but not the crude RA extract, still markedly increased the sleeping time induced by pentobarbital (Fig. 2); (3) the analgesic effect of Dannanxing and Zhinanxing, but not the crude RA extract, was shown by the increase of tail flick reflex latency after 2-, 3-days of consecutive administration and until 4-days after cessation of administration (Fig. 3). These preferential sedative and analgesic effects of Dannanxing and Zhinanxing may account for the better clinical therapeutic application; (4) the neurotoxic actions including abnormal change of stpy-1 episodes of locomotor activities (Fig. 1C) and motor equilibrium dysfunction (Fig. 4) were found only in the mice treated with the crude RA extract. These findings indicate that the art of processing procedures significantly enhanced the pharmacological efficacies and markedly decreased the toxicological effects of the crude RA. In addition, more than thirty types of compounds have been isolated from RA (the rhizome of *Pinellia pedatisecta* Schott), including beta-sitosterol, alkaloids, dipeptides, and amino acids (Qin *et al.*, 1983, 1984, 1995; Wang *et al.*, 1997). It has been indicated that some of these compounds exhibit marked biological activities (Aguirre-Hernandez *et al.*, 2007; Ashidi *et al.*, 2010; Jayaprakasha *et al.*, 2010; Ostrovskaya *et al.*, 1999). Whether, the main active components of processing RA extracts in pharmacological efficacies and the crude RA extract in neurotoxicological effects have not

been identified and remain to be elucidated in future.

The exposure to the crude RA extract (regimen or skin contact) causes many severe toxic effects, including burning feeling in the gullet, paralysis in tongue, mucosa necrosis, edema, asphyxia, and disorder of the mental development as indicated in Pharmacopoeia of China (2000) and other reports (Wu *et al.*, 1994). However, the neurotoxic effect induced by crude RA is still unclear. Moreover, the cerebellum region of brain offers several unique advantages as a model system for motor coordination. If this region is damaged, it can produce behavioral alterations or dysfunctions in stereotype-1 episodes and rotarod performance (Huang *et al.*, 2007; Marquis *et al.*, 1989). The present work showed that ICR mice treated with the crude RA extract for 3 consecutive days could increase parameter of stereotype-1 episodes of locomotor activities and dysfunction of motor equilibrium performance as revealed by the decreased retention time on a rotating rod. After 18 days cessation of administration, these abnormal effects could still be appeared (Fig. 1C and Fig. 4). Furthermore, nitric oxide (NO_x) is an important signaling molecule (a diffusible multifunctional second messenger), which acts as a double-edged sword and first describes in mammals. It not only mediates several physiological functions, but also regulates many pathological processes involved in the regulation of vascular contraction, immune defense against infectious agents, and neurotransmission (Estevez and Jordan, 2002; Moncada *et al.*, 1991). Recent studies have indicated that it can either enhance or inhibit NO_x production associated with the mercurial

compounds-induced neurotoxicity (Huang *et al.*, 2007 and 2008b). In the present study, we have found that the oral administration with the crude RA extract in mice for 3 consecutive days significantly decreased NO_x levels of whole blood, which still persisted at a lower level after 18 days cessation of administration (Fig. 5). These results implicate that the crude RA could inhibit NO_x levels, which might be responsible, at least in part, for inducing the sustained neural behavior dysfunction (abnormalities of both stereotype-1 episodes of locomotor activities and motor equilibrium performance).

Oxidative stress is the result of an imbalance in the pro-oxidant/anti-oxidant homeostasis and implicating in a wide variety of functional damage and disease processes, including cell death, diabetes mellitus, or tissue injury within the central and peripheral nervous system (Castoldi *et al.*, 2001; Kaneto *et al.*, 2005). Many studies have indicated that oxidative stress can produce toxic effects and cause the cellular disruption, the deterioration of brain functions or pathological injury after exposure to toxic chemicals or drugs (Chen *et al.*, 2006; Inoue *et al.*, 2004; Loh *et al.*, 2006). In addition, recent studies of oxidative stress-induced damages were closely paralleled with the inhibition of Na^+/K^+-ATPase activities (Huang *et al.*, 2007; Rodrigo *et al.*, 2007). The membrane bound Na^+/K^+-ATPase is one of the important ion pumps which are crucial for generation and maintenance of Na^+ and K^+ gradients between the intracellular and extracellular milieus, and a prerequisite for basic cellular and specialized organic functional homeostasis of the nerve system (Cheng *et al.*, 2005; Xie *et al.*, 2003). The

inhibition of this enzyme activity by various insults, such as toxic chemicals, drugs or acoustic trauma can induce membrane depolarization, excessive Ca^{2+} entry into neuron, lead to suppression of neuronal and excitatory transmission, and result in the pathological and physiological disorders or neurodegenerative diseases (Balestrino *et al.*, 1999; Yu, 2003). However, the role of Na^+/K^+-ATPase activities in the crude RA-induced neurotoxicity is not clearified. The results in this study showed that the crude RA extract could enhance the plasma lipid peroxidation production (as an indicator for ROS formation)(Fig. 6). It also significantly inhibited the Na^+/K^+-ATPase activities in the cerebral cortex by treatment with the crude RA extract, but not with Dannanxing extract (Fig. 7). Therefore, these findings implicate that ROS generation and inhibition of the Na^+/K^+-ATPase activities were associated with the crude RA-induced abnormalities of locomotor activities and motor equilibrium dysfunction and suggest that oxidative stress and Na^+/K^+-ATPase activities were involved in the toxic mechanism by the crude RA-induced neurotoxicity.

In conclusion, our results provide the pharmacological basis of enhancing the therapeutic effects and decreasing the toxic actions of the crude Rhizoma Arisaematis by the appropriate processing (Fig. 8). Moreover, we also demonstrate the toxicological basis of the crude Rhizoma Arisaematis in mice. The signaling of oxidative stress/NO/Na^+-K^+-ATPase may play an important role, at least in part, in the underlying mechanisms of the neurotoxicological actions the crude Rhizoma Arisaematis.

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Figure legends:

Fig. 1. Effects of Rhizoma Arisaematis (RA) on the spontaneous locomotor activities in mice. Mice were administered with water extracts of the three kinds of RA (processed by alumen plus ginger juice (Zhinanxing), by bile juice (Dannanxing), or crude RA, respectively) (1 g/kg/day, oral application by gavage) or distilled water (control group) for 3 consecutive days. Spontaneous total movements were recorded and analyzed as described in the Materials and Methods. The locomotor activities of all groups were recorded: ambulatory distances (A), jump (B), stereotype-1 episodes (C), and vertical-plane entries (D). All data are presented as mean ± S.E. (n = 8 for each group). $^{*}p < 0.05$ as compared with control group.

Fig. 2. Analysis of prolongedation of pentobarbital-induced sleeping time by the three kinds of Rhizoma Arisaematis (RA) extracts in mice. Mice were administrated with water extracts of the three kinds of RA (processed by alumen plus ginger juice (Zhinanxing), by bile juice (Dannanxing), or crude RA, respectively) (1 g/kg/day, oral application by gavage) for 3 consecutive days. Sleeping time was measured every day during consecutive 3 days administration, and also after 4 days, 11 days, and 18 days cessation of administration as described in the Materials and Methods. All data are presented as mean ± S.E. (n = 8). $^{*}p < 0.05$ as compared with control group, $^{\#}p < 0.05$ as compared with RA group, and $^{\&}p < 0.05$ as compared with Zhinanxing group.

Fig. 3. Influence of Rhizoma Arisaematis (RA) on the thermal induced hyperalgesia in mice.

Mice were orally administrated with either distilled water (control) or with water extracts of the three kinds of RA (processed by alumen plus ginger juice (Zhinanxing), by bile juice (Dannanxing), or crude RA, respectively) (1 g/kg/day) for 3 consecutive days. Tail flick reflex testing expressed as the thermal withdrawal latencies was performed as described in the Materials and Methods. Data are presented as mean ± S.E. (n = 8 for each group). $^{*}P < 0.05$ as compared with control group.

Fig. 4. Effects of Rhizoma Arisaematis (RA) on the motor equilibrium performance testing in mice. Mice were administered with either distilled water (control) or water extracts of the three kinds of Rhizoma Arisaematis (processed by alumen plus ginger juice (Zhinanxing), by bile juice (Dannanxing), or crude RA, respectively) (1 g/kg/day, oral application by gavage) for 3 consecutive days. Equilibrium retention times on rotating rod (60rpm) were recorded and analyzed as described in the Materials and Methods. All data are presented as mean ± S.E. (n = 8 for each group). $^{*}p < 0.05$ as compared with control group.

Fig. 5. Changes of nitric oxide (NO_x) levels of whole blood in mice treated with the crude Rhizoma Arisaematis (RA) extract. Mice were orally administrated with either distilled water (control) or crude RA extract (1 g/kg/day) for 3 consecutive days. Whole blood sample were acquired, de-proteinized and determined of NO_x levels (NO_2^- and NO_3^-) as described in the Materials and Methods. All data are presented as mean ± S.E. (n = 8 for each group). $^{*}P <$ 0.05 as compared with control group.

Fig. 6. Lipid peroxidation (LPO) levels of plasma in crude Rhizoma Arisaematis (RA) treated mice. Mice were orally gavaged with crude RA extracts (0.33 and 1 g/kg/day) for 3 consecutive days, and LPO levels of plasma were determined as described in the Materials and Methods. All data are presented as mean ± S.E. (n = 8 for each group). $^{*}p < 0.05$ as compared with control group.

Fig. 7. Suppression of Na^{+}/K^{+}-ATPase activities in mice. (A).Whole blood sample of mice, which were treated with crude Rhizoma Arisaematis (RA) extracts (0.33 and 1 g/kg/day) for 3 consecutive days, were collected after 4 days cessation of administration and analyzed Na^{+}/K^{+}-ATPase and Mg^{2+}-ATPase activities as described in the Materials and Methods. (B). Changes of Na^{+}/K^{+}-ATPase activities of the cerebral cortex of mice treated with RA extracts (processed by bile juice (Dannanxing), or crude RA, respectively) were determined. The cerebral cortex of normal mice were isolated, homogenized, and then equal volume of samples were treated with different concentrations of Dannanxing or RA extracts and then Na^{+}/K^{+}-ATPase activities were assayed as described in the Materials and Methods. Data in A are presented as mean ± S.E. (n = 8 for each group). Results shown in B are presented as mean ± S.E. for three independent experiments with triplicate determination. $^{*}p < 0.05$ as compared with control group.

Figure 8. Schematic diagram of the processed Rhizoma Arisaematis indeed possessed not only enhanced pharmacological efficacies but also attenuated toxicological effects.

Fig.1

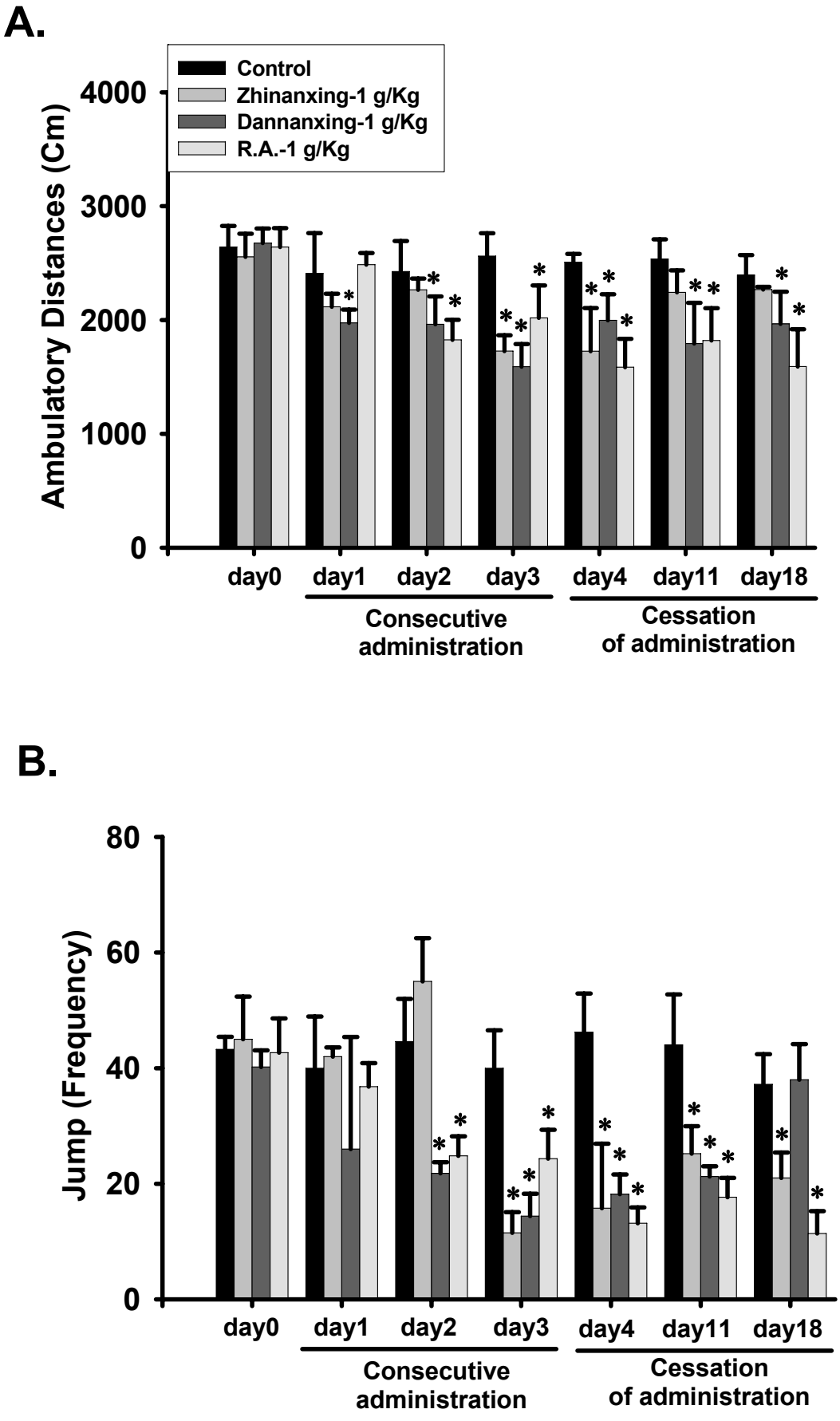
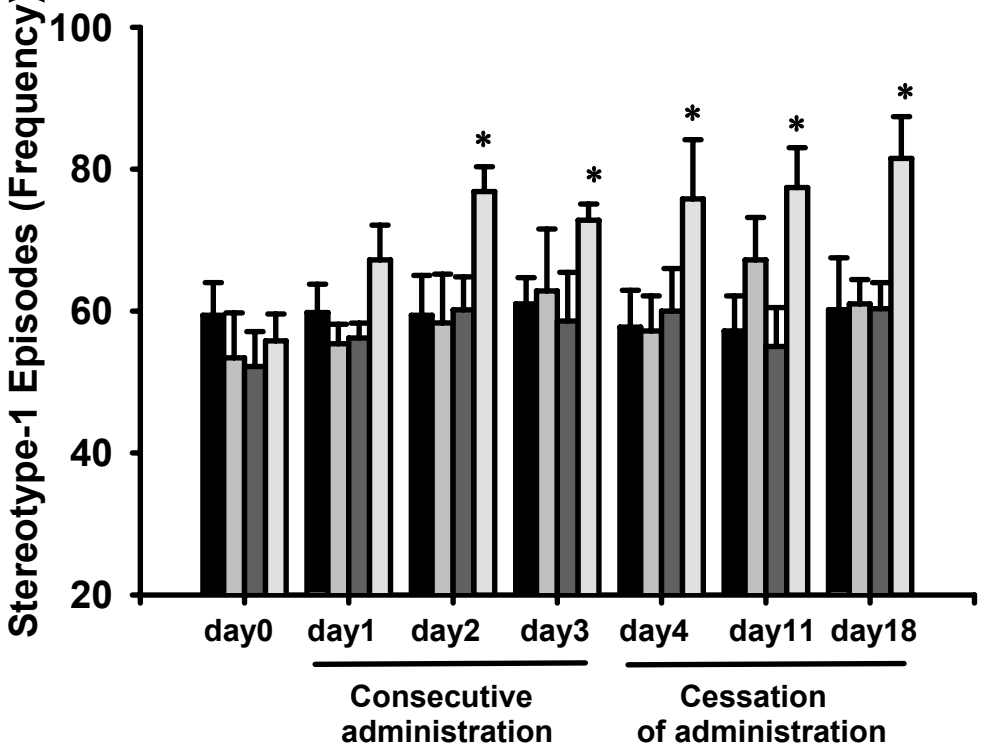

A.
Control
Zhinanxing-1 g/Kg
Dannanxing-1 g/Kg
R.A.-1 g/Kg
Ambulatory Distances (Cm)
4000
3000
2000
1000
0
day0
day1
day2
day3
day4
day11
day18
Consecutive administration
Cessation of administration
B.
Jump (Frequency)
80
60
40
20
0
day0
day1
day2
day3
day4
day11
day18
Consecutive administration
Cessation of administration

Fig.1

C.


Stereotype-1 Episodes (Frequency)
100
80
60
40
20
day0
day1
day2
day3
day4
day11
day18
Consecutive administration
Cessation of administration

D.

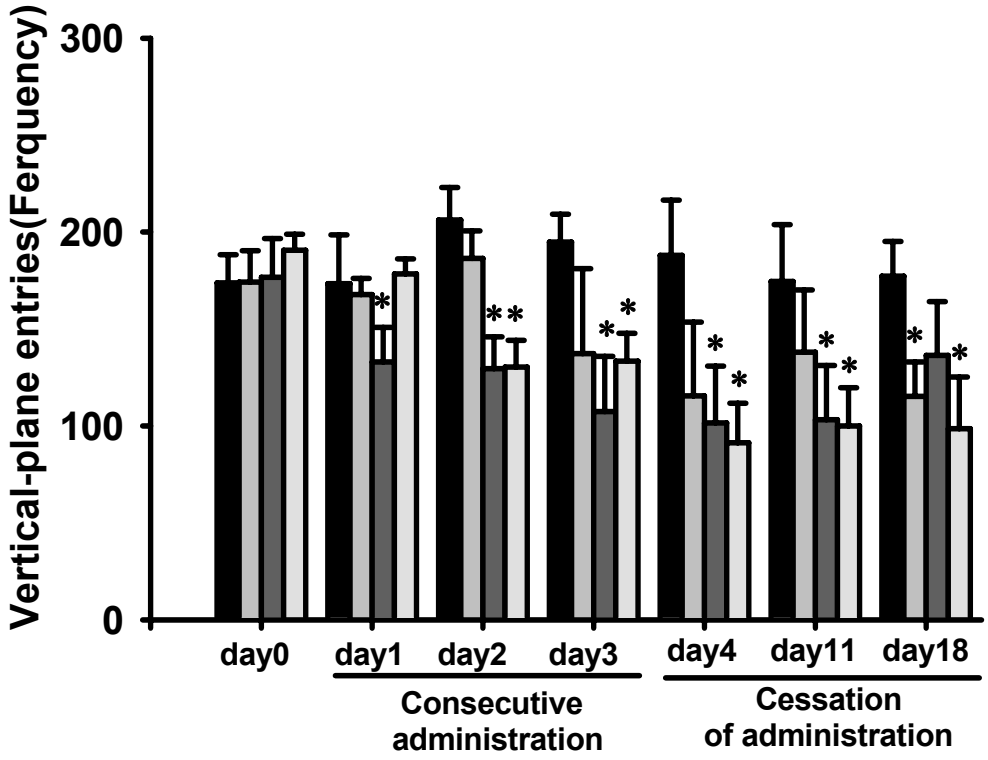

Vertical-plane entries(Ferquency)
300
200
100
0
day0
day1
day2
day3
day4
day11
day18
Consecutive administration
Cessation of administration

Fig.2

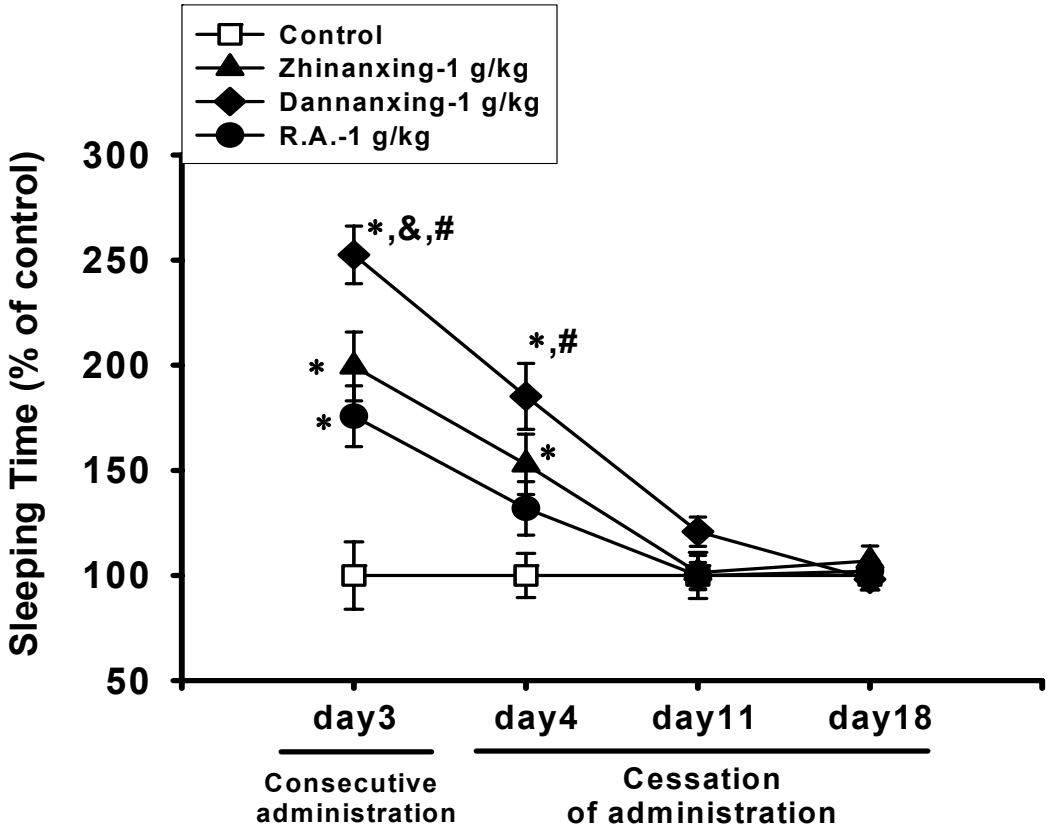

Control
Zhinanxing-1 g/kg
Dannanxing-1 g/kg
R.A.-1 g/kg
Sleeping Time (% of control)
300
250
200
150
100
50
*,&,#
*,#
*
*
*
day3
day4
day11
day18
Consecutive administration
Cessation of administration

Fig.3

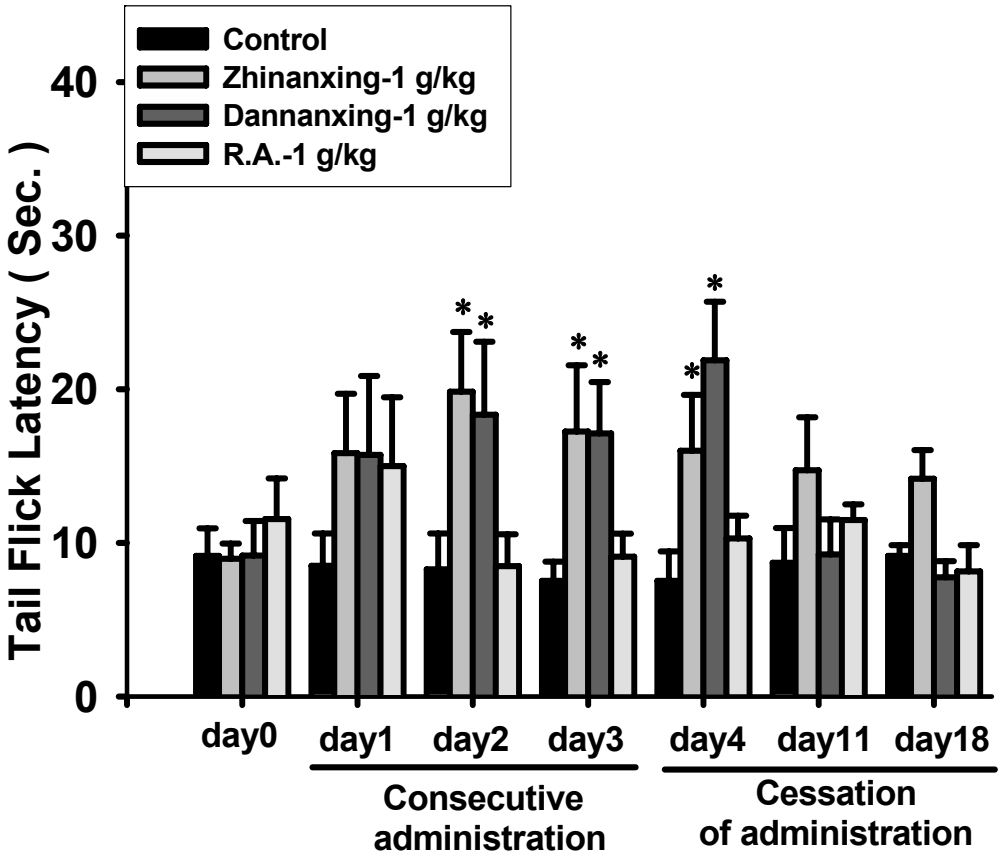

Control
Zhinanxing-1 g/kg
Dannanxing-1 g/kg
R.A.-1 g/kg
Tail Flick Latency (Sec.)
40
30
20
10
0
day0
day1
day2
day3
day4
day11
day18
Consecutive administration
Cessation of administration

Fig.4

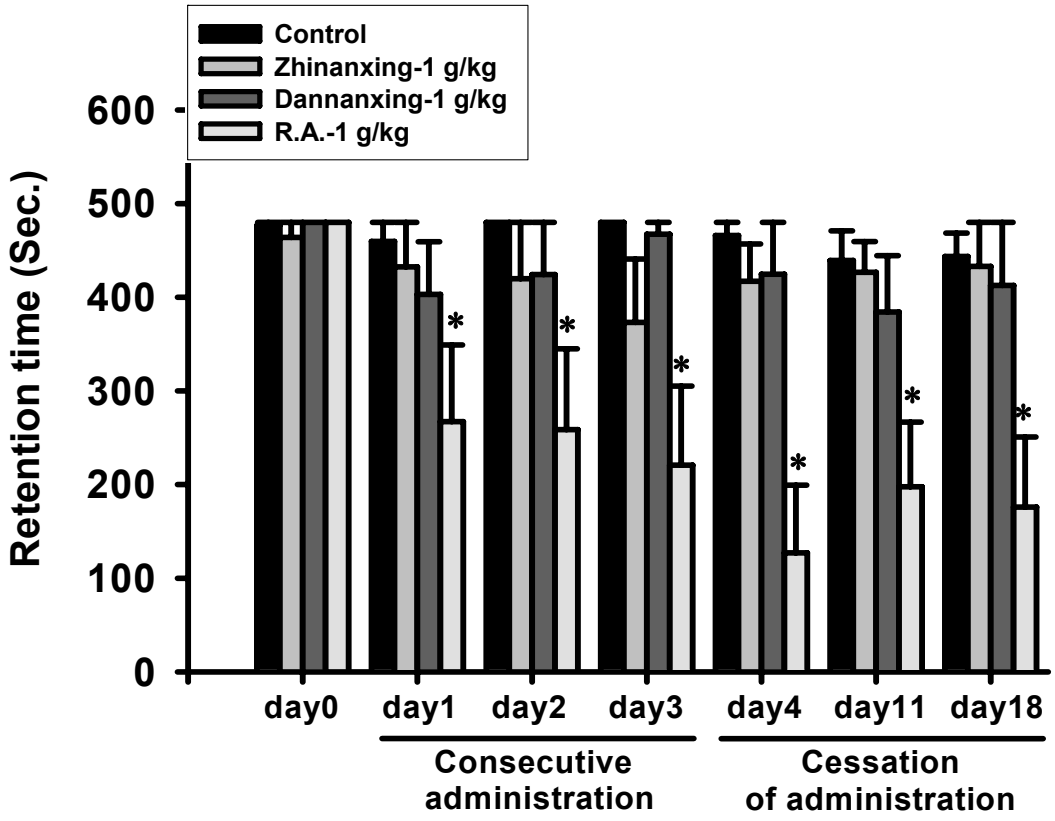

Control
Zhinanxing-1 g/kg
Dannanxing-1 g/kg
R.A.-1 g/kg
Retention time (Sec.)
600
500
400
300
200
100
0
*
day0
day1
day2
day3
day4
day11
day18
Consecutive administration
Cessation of administration

Fig.5

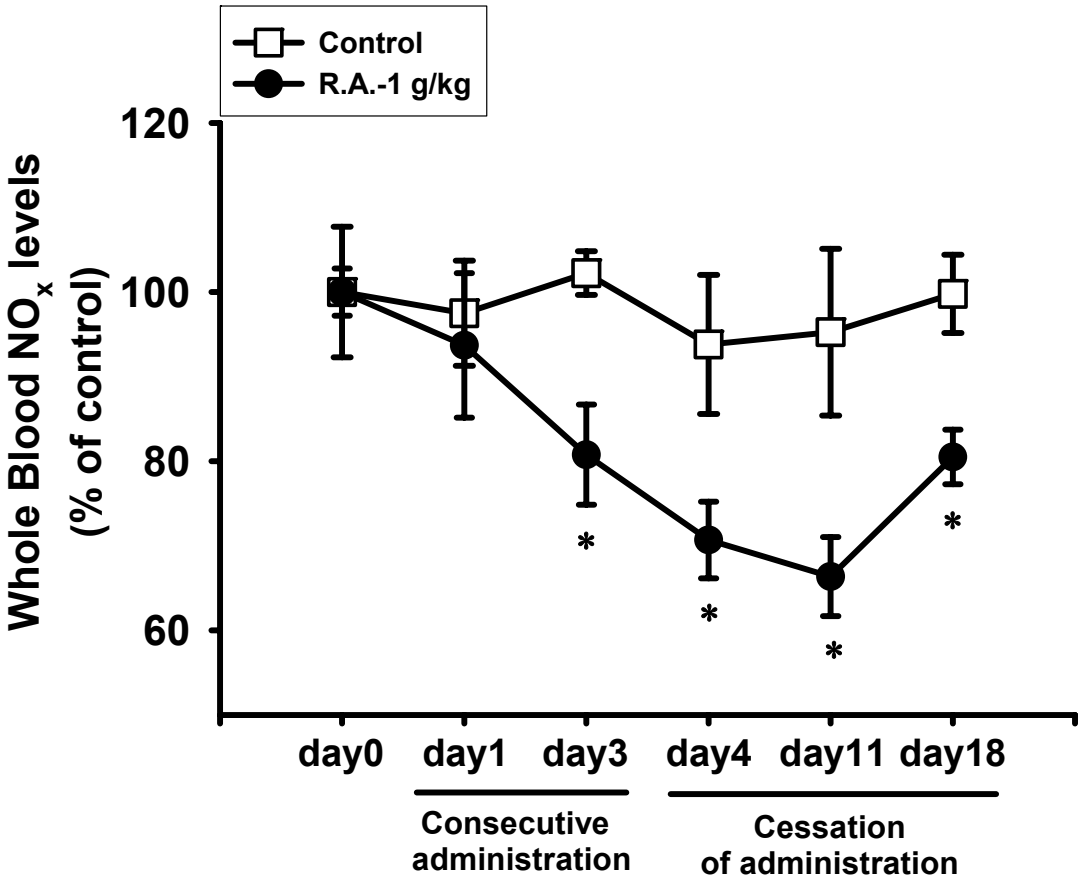

Control
R.A.-1 g/kg
Whole Blood NO_x levels
(% of control)
120
100
80
60
*
*
*
*
day0
day1
day3
day4
day11
day18
Consecutive
administration
Cessation
of administration

Fig.6

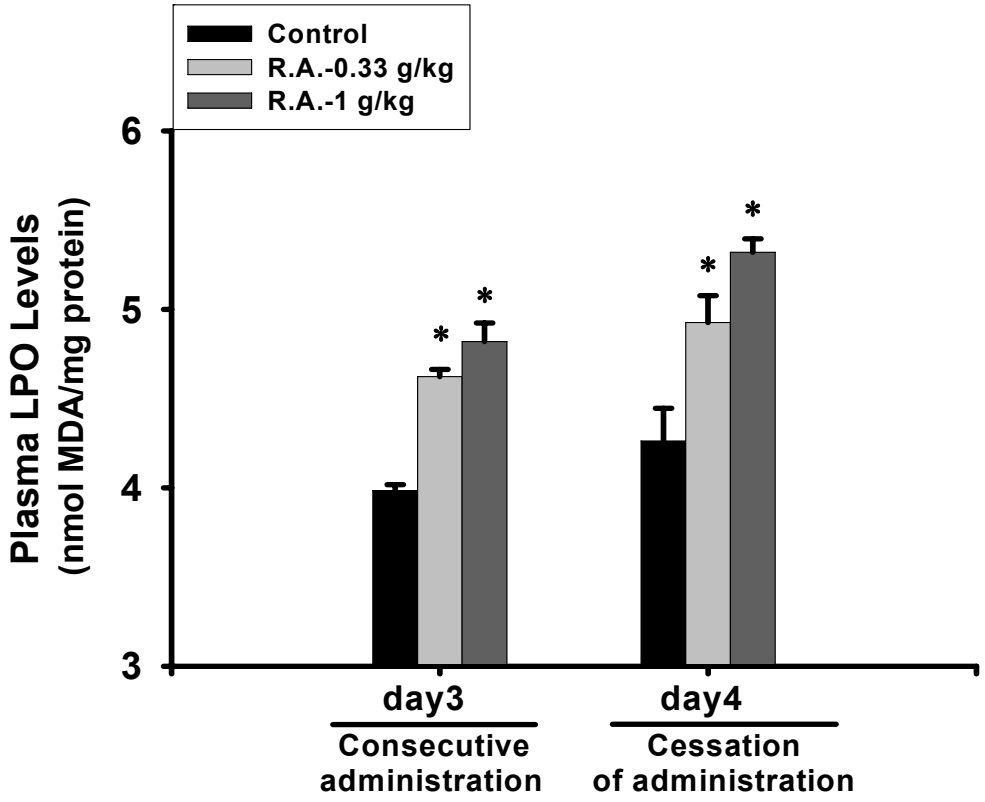
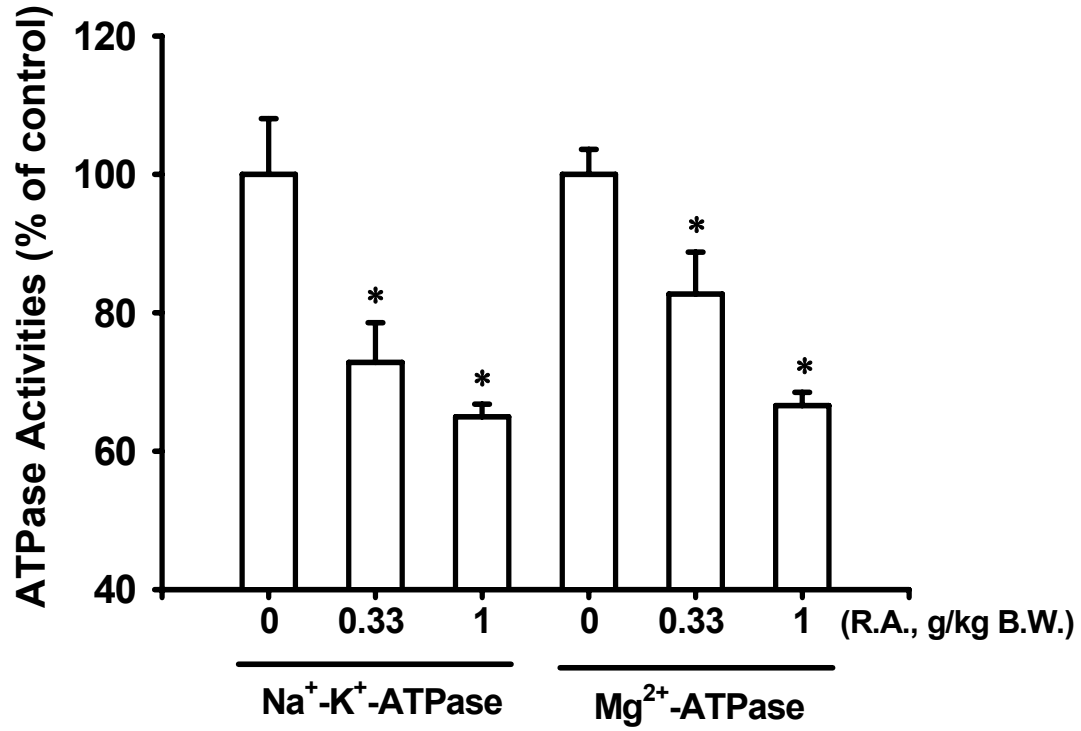

Control
R.A.-0.33 g/kg
R.A.-1 g/kg
Plasma LPO Levels
(nmol MDA/mg protein)
6
5
4
3
*
*
*
*
*
day3
day4
Consecutive
administration
Cessation
of administration

Fig.7

A.


ATPase Activities (% of control)
120
100
80
60
40
0
0.33
1
0
0.33
1
(R.A., g/kg B.W.)
Na+-K+-ATPase
Mg2+-ATPase
*

B.

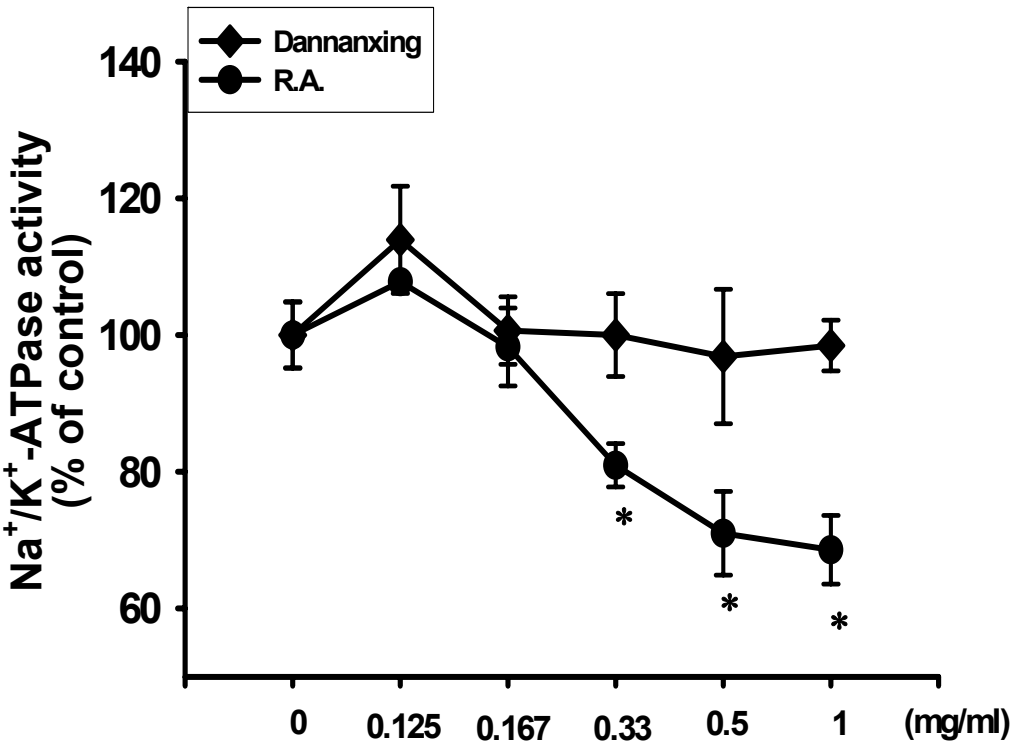
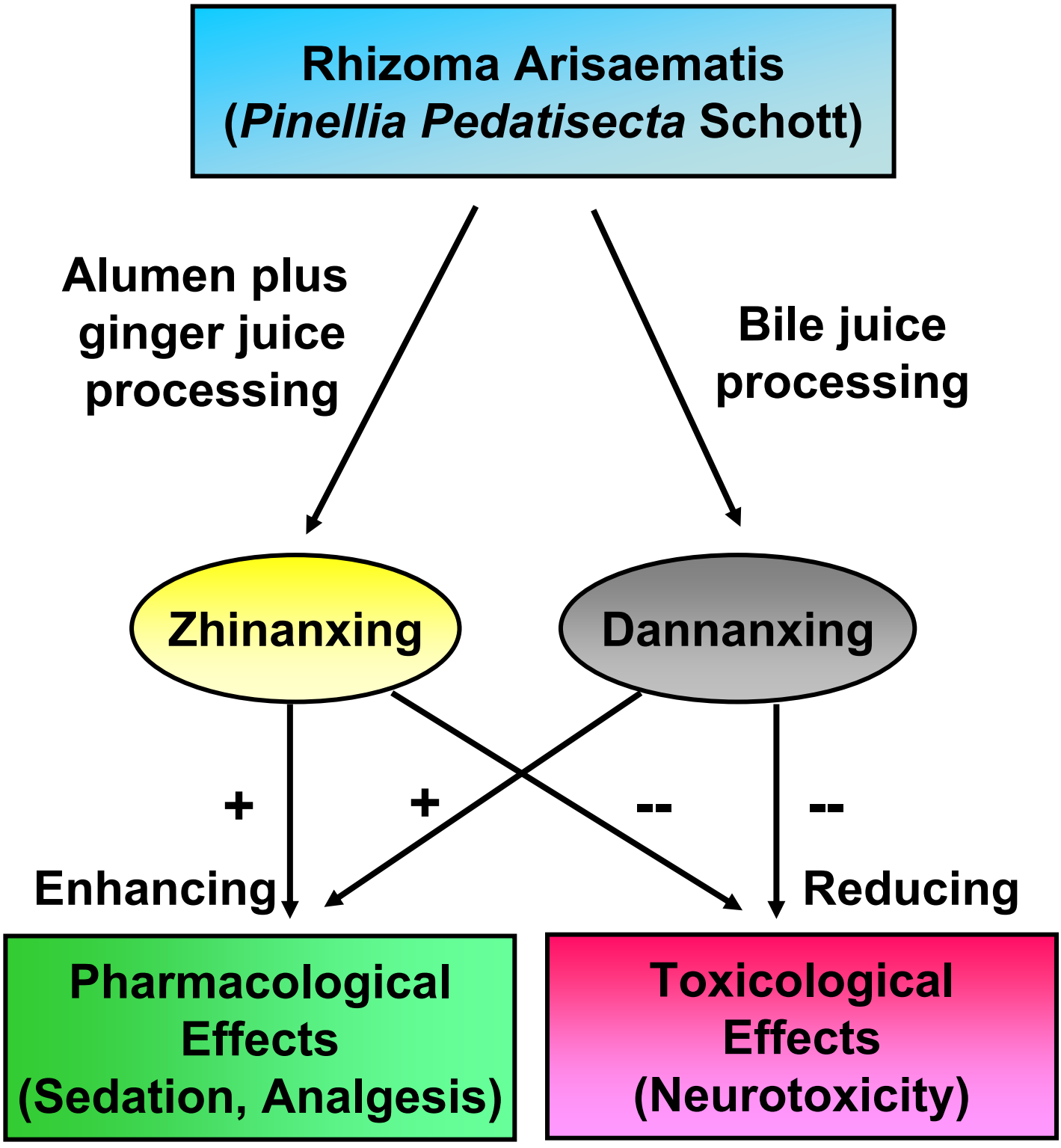

Dannanxing
R.A.
Na+/K+-ATPase activity
(% of control)
140
120
100
80
60
0
0.125
0.167
0.33
0.5
1
(mg/ml)
*

Fig.8


Rhizoma Arisaematis
(*Pinellia Pedatisecta* Schott)
Alumen plus ginger juice processing
Bile juice processing
Zhinanxing
Dannanxing
+
+
--
--
Enhancing
Reducing
Pharmacological Effects (Sedation, Analgesis)
Toxicological Effects (Neurotoxicity)