The effects of Acorus tatarinowii Schott on 5-HT concentrations, TPH2 and 5-HT1B expression in the dorsal raphe of exercised rats

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physcione (PubChem CID:10639)
emodin (PubChem CID:3220)
trans-Galbacin (PubChem CID:442873)
3,4,5-Trimethoxybenzaldehyde oxide (PubChem CID:6875502)
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ABSTRACT

Ethnopharmacological relevance: Acorus tatarinowii Schott (Shi Chang Pu) belongs to the family of Acoraceae. The plant is used as an important herb for prolonging life many years in traditional Chinese medicine. It is an ancient herbal tonic nutrient and can be used as anti-fatigue medicine. However, the effects of Acorus tatarinowii Schott on the endurance exercise in relation to central nervous system have not yet been clarified. In this study, the effects of Acorus tatarinowii Schott on treadmill running endurance, 5-HT concentrations, TPH2, 5-HT1B expression in the dorsal raphe of exercised rats were investigated.

Materials and methods: Sixty adult male Sprague–Dawley rats were randomly divided into six groups: the normal group, the exercise group, the exercise and the rhizomes of Acorus tatarinowii Schott (ATS)(1 mg/ kg)-treated group, the exercise and ATS (10 mg/kg)-treated group, the exercise and ATS (100 mg/kg)-treated group, the exercise and caffeine (10 mg/kg)-treated group. The effects of Acorus tatarinowii Schott on endurance exercise were determined by the time to exhaustion during treadmill exercise. The detection of 5-HT concentrations in the dorsal raphe was performed by HPLC analysis. The levels of TPH2, 5-HT1A and 5-HT1B expression were measured by western blot analysis and real-time PCR.

Results: We found Acorus tatarinowii Schott could prolong the time to exhaustion in treadmill exercise and suppress the exercise-induced increase of 5-HT synthesis, TPH2 mRNA and protein expression and prevent the exercise-induced decrease of 5-HT1B mRNA and protein expression in the dorsal raphe. Acorus tatarinowii Schott was as effective as caffeine in prolonging the exhaustion time in treadmill running and in decreasing the exercise-induced increase of 5-HT synthesis and TPH2 mRNA and protein expression and in preventing the exercise-induced decrease of 5-HT1B mRNA and protein expression in the dorsal raphe.

Conclusions: The results indicated that the effects of Acorus tatarinowii Schott in inhibiting the exercise-induced synthesis of 5-HT and TPH2 expression and in preventing the exercise-induced decrease of 5-HT1B expression in the dorsal raphe might be the anti-fatigue mechanism of Acorus tatarinowii Schott.

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1. Introduction

Physical exercise is often terminated not due to muscle fatigue but to the increase in serotonin or 5-hydroxytryptamine (5-HT) concentrations in the brain during prolonged exercise (Cotel et al., 2013; Falavigna et al., 2012). Increased concentrations of brain 5-HT during sustained physical activity could hasten the onset of fatigue (Soares et al., 2007) while decreasing 5-HT concentration could delay the time to fatigue (Seo et al., 2011). Serotonin is modulated by many factors involved in intrinsic regulation of central 5-HT neurotransmission, which include tryptophan hydroxylase (TPH), serotonergic type 1B (5-HT1B) inhibitory autoreceptor (Foley et al., 2006). The second isoform of TPH (TPH2) is considered to be the rate-limiting enzyme in 5-HT synthesis (Murphy et al., 2008). 5-HT1B inhibitory autoreceptor, upon stimulation, inhibit local synthesis and the release of 5-HT (Adell et al., 2001).

Acorus tatarinowii Schott (Shi Chang Pu) is a herb widely used in traditional Chinese medicines. It can not only be anti-dementia, effective in improving learning and memory, be antidepressant, but also be sedative, anticonvulsant and antiepileptic (Han et al., 2013; He and Li, 2008; Liao et al., 2005; May et al., 2013). Acorus tatarinowii Schott is also used as an important herb for prolonging life in traditional Chinese medicines. Shen Nong’s herbal classic...
listed it as a herb of top grade. It is an ancient herbal tonic nutrient (Dou, 1990). The extract of *Acorus tatarinowii* Schott could obviously prolong the time to exhaustion in forced swimming and significantly increase sugar reserve in muscle and liver (Xiong et al., 2009a, 2009b), effectively inhibited exercise-induced decrease of red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) (Xiong et al., 2009a, 2009b), exhibited the strong anti-fatigue effect on exercised mice (Zhu et al., 2009a, 2009b). The volatile extracts, n-BuOH extracts, remains of n-BuOH extraction, and H2O extracts of the rhizomes of *Acorus tatarinowii* Schott could significantly delay exercise fatigue symptoms and improve exercise capacity of mice (Zhu et al., 2009a, 2009b). However, the effects of *Acorus tatarinowii* Schott on the endurance exercise in relation to central nervous system have not yet been clarified. In this study, the effects of *Acorus tatarinowii* Schott on treadmill running endurance, 5-HT concentrations, TPH2, 5-HT1B expression in the dorsal raphe of exercised rats were investigated.

2. Materials and methods

2.1. Plant material and preparation of plant extract

The rhizomes of *Acorus tatarinowii* Schott (ATS) in this experiment were bought from Kunming TCMs CO., Ltd. (Kunming, Yunnan, China) and identified by Prof. Ning-Hua Tan from Kunming Institute of Botany, Chinese Academy of Sciences. Voucher specimen (No. 20070014) was deposited at the Constitutional Research Center, School of Sports Science, and Jinggangshan University.

It has been well documented that asarone is a main component of the *Acorus tatarinowii* Schott. We used the method of high performance liquid chromatography to quantify the cis-asarone and trans-asarone. The rhizomes of *Acorus tatarinowii* Schott in this experiment contains 1.00–1.50% cis-asarone and 0.20–0.48% trans-asarone of them.

The rhizomes of *Acorus tatarinowii* Schott (180 g) were immersed in water at room temperature for 24 h and then extracted with boiling water for 2 h three times. After lyophilization, the 18 g extract was obtained, which was diluted with saline, filtered through a 0.50 µm syringe filter and stored at 4 °C.

2.2. Animals and treatment

Adult male Sprague–Dawley rats 230 ± 11.32 g in weight (grade SPF and 5 weeks of age) were obtained from Hunan Lake King of laboratory animal Co. Ltd, Changsha, Hunan, China. Animals were housed under the controlled conditions of temperature (25–26 °C), humidity (40%), a light/dark cycle (12 h/12 h) with food and water at will. Animals were allowed to acclimatize to the laboratory before the commencement of the experiment. The rodent license of the laboratory (no.SCKX (Xiang) 2011–0003) was issued by the Hunan Province Laboratory Animal Care and Use Committee. All experiments were carried out according to the general principles of laboratory animal care (NIH publication#85-23, revised in 1985) and the Guidance Suggestions for the the Hunan Province Laboratory Animal Care and Use Committee.

The animals were randomly divided into six groups (n=10 in each group): the normal group, the exercise group, the exercise and ATS (1 mg/kg)-treated group, the exercise and ATS (10 mg/kg)-treated group, the exercise and ATS (100 mg/kg)-treated group, the exercise and caffeine (10 mg/kg)-treated group. Rats of the ATS-treated groups were injected by the intragastric gavage (ig) once per day with an aqueous extract of *Acorus tatarinowii* Schott at the respective one time dose. Rats of the caffeine-treated group received 10 mg/kg caffeine by the same method. Rats of the normal group and the exercise group were administrated with the same volume of saline by ig. The volume of each intragastric gavage was 2 ml per rat and the intragastric gavage was performed at 2 h before the start of treadmill running.

2.3. Treadmill exercise protocol.

The physical exercise load applied in this study took the form of treadmill exercise on a motor-driven treadmill. Rats in the training groups had been doing exercises through running on a treadmill with 0° of inclination for 6 consecutive days. The normal groups were left on the treadmill with no running for 30 min. The exercise duration consisted of forced running at a speed of: 10 m/min for 10 min, 13 m/min for another 10 min, and 16 m/min for the last 10 min from the first day to the third day, and 15 m/min for 10 min, 18 m/min for another 10 min, and 23 m/min for the last 10 min from the fourth day to the sixth day.

2.4. Determination of exhaustion time and collection of the dorsal raphe sample

On the 7th day of the experiment, the time to exhaustion for treadmill exercise was determined for the exercise groups. Time to exhaustion is defined as the time between the commencement of exercise and the first occurrence of the experimental animals failing to keep up with the treadmill machine for a period of 3 min or more. The speed used for measurement of the time to exhaustion was 18 m/min for 2 min, 21 m/min for 2 min, 24 m/min for 2 min, and then 26 m/min until exhaustion. Immediately after the determination of the exhaustion times all rats were sacrificed to remove the dorsal raphe tissues (–7.30 to –8.30 mm posterior to bregma) for further processing.

2.5. HPLC analysis of 5-HT concentration in the dorsal raphe

The dorsal raphe tissues were sonicated in ice-cold 0.1 M HClO4 containing 0.01% EDTA. The supernatant collected after centrifugation at 10,000g for 5 min was injected (10 µl) into the HPLC system (Bioanalytical Systems Inc., West Lafayette, USA) equipped with a pump (PM80), ESA electrochemical detector (Coulombich III) with glassy carbon working electrode (5041 cell, 350 mV), and a Rheodyne injector. A C18, ion pair, analytical column (2.1 mm × 250 mm; Zorbax Eclipse Plus; Agilent, USA), with a particle size of 5 µm and pore of 95 Å was used for separating 5-HT. The flow rate was 0.7 ml/min and the electrochemical detection was performed at +0.35 V. The composition of the mobile phase was 200 mg OSA, 50 µl EDTA, 5% MeOH, and 0.1 M orthophosphoric acid.

2.6. Western blot analysis

The dorsal raphe homogenate was centrifuged at 13,000g for 15 min at 4 °C and the supernatant was collected. Protein concentration was measured by Bio-Rad Protein Assay (BioRad, Hercules, CA, USA). Sample proteins were separated on SDS-polyacrylamide gels, and then transferred to a polyvinylidene difluoride membrane, blocked by 5% non-fat milk and incubated overnight with the primary antibodies anti-TPH2 (1:1000; sc-134775; Santa Cruz, CA, USA) or anti-5-HT1B (1:500; ab13896; Abcam, Cambridge, UK). The membrane was washed twice and then incubated for 1 h with secondary antibodies (anti-rabbit IgG for TPH2 & 5-HT1B, 1:10,000; ab97060, Abcam, Cambridge, UK), and the bound antibody was detected by using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). For the gel loading control, membranes were reprobed with a monoclonal anti-GAPDH antibody (Santa Cruz,
3.1. The effect of Acorus tatarinowii Schott

The effect of the different doses of Acorus tatarinowii Schott on the time to exhaustion by treadmill exercise was 68.80 ± 9.11 min in the exercise and ATS (100 mg/kg)-treated group, and it was 68.20 ± 8.78 min in the exercise and caffeine (10 mg/kg)-treated group (Fig. 1). The results showed that Acorus tatarinowii Schott (100 mg/kg) was just as effective as caffeine (10 mg/kg) in prolonging the time to exhaustion during treadmill exercise.

3. Results

3.1. The effect of Acorus tatarinowii Schott (ATS) on the time to exhaustion by treadmill exercise

The effect of the different doses of Acorus tatarinowii Schott on the time to exhaustion of rats is presented in Fig. 1. The mean exhaustion time for forced treadmill exercise in a dose-dependent way.

3.2. The effect of Acorus tatarinowii Schott (ATS) on 5-HT concentrations in the dorsal raphe of exercised rats

The effect of Acorus tatarinowii Schott (ATS) on 5-HT concentrations in the dorsal raphe is presented in Fig. 2. 5-HT concentrations in the dorsal raphe were 14.65 ± 1.71 nmol/g tissue in the exercise and ATS (10 mg/kg)-treated group, 14.65 ± 1.71 nmol/g tissue in the exercise and ATS (100 mg/kg)-treated group, and 18.88 ± 1.09 nmol/g tissue in the exercise and ATS (10 mg/kg)-treated group. The results showed that treadmill running increased the 5-HT levels in the dorsal raphe, and Acorus tatarinowii Schott suppressed the exercise-induced increase of 5-HT levels in a dose-dependent way.

3.3. The effect of Acorus tatarinowii Schott on the levels of TPH2 mRNA and protein expression in the dorsal raphe of exercised rats

The effect of Acorus tatarinowii Schott on the levels of TPH2 mRNA expression in the dorsal raphe for treadmill running was presented in Fig. 3. The levels of TPH2 mRNA in the dorsal raphe for the ATS (100 mg/kg)-treated group increased significantly higher than those of the control group (P < 0.01). The rats of the exercise group exhibited significantly higher levels of TPH2 mRNA in the dorsal raphe than those of the exercise and ATS (10 mg/kg)-treated and ATS (100 mg/kg)-treated

### Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Accession no.</th>
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<td>TPH2</td>
<td>GGTGCTTGATTTGGTTCGCTGTTG</td>
<td>CCGGTTGCTTGCTGCTC</td>
<td>NM_173391</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>GAACCAAGTCAAAATGGCGAGTC</td>
<td>CACGGGAGATGAAGAAGGG</td>
<td>NM_010482</td>
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</tbody>
</table>
TPH2 mRNA and protein expression levels in a dose-dependent way, exercise were presented, respectively in Fig. 5, and Fig. 6. The levels of TPH2 mRNA and protein expression in the dorsal raphe for treadmill exercise were increased. Acorus tatarinowii Schott suppressed the exercise-induced decrease of the level of 5-HT1B protein expression in the dorsal raphe (Fig. 6). The results showed that treadmill exercise decreased the levels of 5-HT1B mRNA and protein expression in the dorsal raphe. A: the control group; B: the exercise group; C: the exercise and ATS (1, 10 mg/kg)-treated group; D: the exercise and ATS (10 mg/kg)-treated group; E: the exercise and ATS (100 mg/kg)-treated group; F: the exercise and caffeine (10 mg/kg)-treated group. Values are mean ± SD. *p < 0.01, compared with A; ††p < 0.01, compared with E.

3.4. The effects of Acorus tatarinowii Schott on the levels of 5-HT1B mRNA and protein expression in the dorsal raphe of exercised rats

The effects of Acorus tatarinowii Schott on the levels of 5-HT1B mRNA and protein expression in the dorsal raphe for treadmill exercise were presented, respectively in Fig. 5, and Fig. 6. The levels of 5-HT1B mRNA in the dorsal raphe in the exercise groups were significantly lower than those of the control group (P < 0.01). The levels of 5-HT1B mRNA in the dorsal raphe in the exercise group were lower than those of the exercise and ATS (10 mg/kg)-treated and ATS (100 mg/kg)-treated group (P < 0.01). The levels of 5-HT1B mRNA in the dorsal raphe in the exercise and ATS (10 mg/kg)-treated were lower than that of the exercise and ATS (100 mg/kg)-treated group (P < 0.01). No significant change was observed for the levels of TPH2 mRNA in the dorsal raphe between the ATS (100 mg/kg)-treated group and the ATS (10 mg/kg)-treated group (P > 0.05). Similar results were obtained by using the method of western blot analysis to detect the level of TPH2 protein expression in the dorsal raphe (Fig 4). The results showed that treadmill running increased the levels of TPH2 mRNA and protein expression in the dorsal raphe, and Acorus tatarinowii Schott suppressed the exercise-induced increase of the TPH2 mRNA and protein expression levels in a dose-dependent way, and Acorus tatarinowii Schott (100 mg/kg) was just as effective as caffeine (10 mg/kg) in suppressing the exercise-induced increase of TPH2 mRNA and protein expression levels.

4. Discussion

This study demonstrated that Acorus tatarinowii Schott extended the time to exhaustion during treadmill running in a dose-dependent manner. Previous research also showed that the extract of the rhizomes of Acorus tatarinowii Schott exhibited strong anti-fatigue effects on exercised mice (Zhu et al., 2009a, 2009b). The volatile extracts, n-BuOH extracts, remains of n-BuOH extraction, and
H₂O extracts of the rhizomes of *Acorus tatarinowii* Schott could significantly delay exercise fatigue symptoms and improve exercise capacity of mice (Zhu et al., 2009a, 2009b). The present results also show that *Acorus tatarinowii* Schott enhanced the endurance ability of rats during treadmill exercise. The precise mechanism of *Acorus tatarinowii* Schott increasing the endurance ability for treadmill exercise has been poorly understood. 5-HT has been suggested to be involved in fatigue induced by strenuous exercise.

It has been suggested that increase of 5-HT concentration in the brain is relevant to the increase in the level of physical fatigue and perhaps to that of mental fatigue as well (Davis et al., 2000). Increase in the level of 5-HT during endurance exercise coincides with the onset of fatigue (Stepto et al., 2011). Exhaustive training can lead to the increase of 5-HT concentrations and the decrease of endurance performance (Caperuto et al., 2009). Inhibition of 5-HT production in the brain could increase endurance exercise performance (Seo et al., 2011). In this study, 5-HT concentrations in the dorsal raphe in the exercise group was significantly higher than the other groups, which supports the viewpoint. Furthermore, *Acorus tatarinowii* Schott treatment was proved to be able to inhibit exercise-induced increase in 5-HT concentrations in the dorsal raphe. The most potent inhibition of *Acorus tatarinowii* Schott on 5-HT concentrations was observed at the dose of 100 mg/kg of the rhizomes *Acorus tatarinowii* Schott. These data suggest that *Acorus tatarinowii* Schott could restrain the increase in 5-HT activity produced by physical activity through increasing local 5-HT1B autoinhibition of 5-HT neurons in the dorsal raphe.

Caffeine is known as an ergogenic aid. Previous research showed that caffeine increased all-out time in exercised rats, and inhibited the exercise-induced elevation in TPH expression (Lim et al., 2001). In this study, we also found that 100 mg/kg of *Acorus tatarinowii* Schott was just as effective as 10 mg/kg caffeine at the exhaustion-time by treadmill running, 5-HT concentrations, TPH2 and 5-HT1B mRNA and protein expression in the dorsal raphe.

There is a two-way regulation of *Acorus tatarinowii* Schott on the central nerve system. It cannot only be anti-dementia, improve learning and memory, be antidepressant, but also be sedative, anticonvulsant and antiepileptic (Han et al., 2013; He and Li, 2008; Liao et al., 2005; May et al., 2013). Medium and low dose of *Acorus tatarinowii* Schott water extracts with oil removed was able to reduce cortical P-glycoprotein expression of multidrug resistance gene product of pentyleneetetrazol kindled rats (Lin et al., 2011). *Acorus tatarinowii* Schott contains quite a lot of organic compounds. Asarone is a main component of the *Acorus tatarinowii* Schott. Cis-asarone delays the onset of muscular fatigue in vitro (Zhu et al., 2013). 5-hydroxymethyl furfural, as a component of the *Acorus tatarinowii* Schott, exhibited a notable anti-fatigue activity in vitro (Zhu et al., 2012). The present study shows that *Acorus tatarinowii* Schott retards fatigue during exercise by inhibition of 5-HT synthesis and TPH2 expression and augmentation 5-HT1B expression. Further study is needed to identify the main active components of *Acorus tatarinowii* Schott which are responsible for the retarding of fatigue in vivo.

### 5. Conclusion

In this research, *Acorus tatarinowii* Schott extended the time to exhaustion during treadmill running in a dose-dependent manner. *Acorus tatarinowii* Schott also retarded fatigue during exercise by inhibiting 5-HT synthesis and TPH2 expression and by increasing 5-HT1B expression in a dose-dependent manner. It suggested that the effect of *Acorus tatarinowii* Schott in inhibiting the exercise-induced synthesis of 5-HT and TPH2 expression and in improving 5-HT1B expression in the dorsal raphe might be the possible anti-fatigue mechanism of *Acorus tatarinowii* Schott. The results from this study confirm the use of *Acorus tatarinowii* in traditional medicine and suggest its therapeutic potency as an antifatigue agent.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.jep.2014.10.026](http://dx.doi.org/10.1016/j.jep.2014.10.026).