

## **SUPPLEMENTARY MATERIAL**

### **Pinellia ternata (Thunb.) Makino Preparation promotes sleep by increasing REM sleep**

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#### **Abstract**

Pinellia ternata (Thunb.) Makino Preparation (PTP) is widely used to treat insomnia in traditional Chinese medicine; however, its specific role is not clear. In this study, PTP was prepared at 3 concentrations. For locomotor activity tests, mice were treated with PTP and evaluated for 14 days. For polygraph recordings, mice were treated for 14 days and recorded after treatment. The main chemical constituents in PTP were identified by Ultra performance liquid chromatography/quadrupole time spectrometry (UPLC/Q-TOF-MS). The results showed that 0.9 g/ml PTP significantly reduced locomotor activity. The effect was related to the time of treatment. PTP reduced wakefulness and increased sleep in mice. Furthermore, PTP promoted sleep by increasing the number of REM sleep episodes with a duration of 64-128s and increasing the number of transitions from NREM sleep to REM sleep and from REM sleep to wakefulness. A total of 17 compounds were identified.

#### **Experimental**

##### ***Animals***

Male SPF C57BL/6J mice (10-12 weeks old, 20-25g) were supplied by the Laboratory Animal Centre of the Chinese Academy of Sciences (Shanghai, China). The animals were housed with free access to food and water at a temperature of 22±0.5°C. The light was automatically controlled on a 12:12 h cycle (lights on at 7:00 AM and off at 7:00 PM). The illumination intensity was approximately 100 lx. The relative humidity was set as 60±2%. All the studies conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimise the number of animals used and their pain and discomfort.

##### ***Drugs and reagents***

PTP was obtained from China Resources Sanjiu (Beijing) Pharmaceutical Co., Ltd. (Beijing, China) (batch number 1207001H) and identified by Xiaochen Chen, the deputy director of the TCM pharmacy of the First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China. Diazepam (DZP) was obtained from Shanghai Xudong Haipu Pharmaceutical Co., Ltd.

(Shanghai, China) (batch number AH120701) and was used as a positive control at a concentration of 0.1 mg/ml. SWFI was obtained from Fuzhou Haiwang Pharmaceutical Co., Ltd. (Fujian, China) (batch number M11073107) and used as a placebo and solvent.

One bag of PTP contained 6 g of PTP granules, which was equal to 6 g of raw PTP. In total, 4.5 bags, 9 bags and 18 bags of PTP granules were dissolved in SWFI and titrated to 60 ml to yield concentrations of 0.45 g/ml, 0.9 g/ml, and 1.8 g/ml (equal to 9g/kg, 18g/kg, and 36g/kg), respectively, and the solutions were stored at 4°C. One tablet of DZP was dissolved in 25ml of SWFI to obtain 0.1 mg/ml DZP liquid.

HPLC-grade acetonitrile was purchased from J.T. Baker Company (J.T. BAKER, USA). HPLC-grade water (18MΩcm) was directly obtained with a Milli-Q purification system (Millipore Co., USA).

### ***Drug extraction***

PTP granules were subjected to cold soaking overnight in methane cyanide: water (3:1, v/v). Then, they were extracted for 45min by ultrasonication and centrifuged for 10min at 11,000 g. The supernatant liquid was filtered through a 0.22-μm microporous membrane and used for testing.

### ***Loco-motor activity test***

To investigate the sedative effect of PTP, the mice were subjected to a locomotor activity test. Twenty mice were randomly divided into the following 5 groups: (1) placebo group (SWFI), mice were intragastrically administered 0.5 ml of SWFI; (2) positive control group (DZP), mice were intragastrically administered 2 mg/kg DZP; (3) PTP group 1 (PTP 1), mice were intragastrically administered 0.45 g/ml PTP at 9g/kg; (4) PTP group 2 (PTP 2), mice were intragastrically administered 0.9 g/ml PTP at 18g/kg; and (5) PTP group 3 (PTP 3), mice were intragastrically administered 1.8 g/ml PTP at 36g/kg. Each dose of PTP and DZP was calculated as follows: the weight of the mouse × 0.02. The choice of drug concentration was based on a previous experiment (MM Fang et al 2016).

Before behaviour recording, all the mice were habituated to the container for 24 h. All the treatments were administered to mice at 19:00 for 14 days. The locomotor activity was continuously recorded from the 1<sup>st</sup> day to the last day. The daily locomotor activity was determined by summing the corresponding 24 h records.

### ***Polygraphic recordings***

Polygraph recordings and vigilance state analysis were applied to test the hypnotic effect of PTP in mice. The test was performed as described previously (YQ Wang et al 2015).

The mice were randomly divided into the following 3 groups: (1) placebo control group (SWFI), mice were intragastrically administered 0.5 ml of SWFI; (2) positive control group (DZP), mice were intragastrically administered 2mg/kg DZP; and (3) PTP group (PTP 2), mice were intragastrically administered 0.9 g/ml PTP at 18g/kg. The concentration of PTP was selected based on the results of the locomotor activity test.

The mice were anaesthetised with chloral hydrate (360 mg/kg, i.p.). Then, electrodes were implanted into the mice for EEG and EMG recordings. Two stainless steel screws (1 mm in diameter) were inserted through the skull above the cortex (front-back, +1.0 mm; left-right, -1.5 mm from bregma or lambda) and served as EEG electrodes. Two insulated, stainless steel, Teflon-coated wires were bilaterally placed into both cowl-muscles and served as EMG electrodes. All the electrodes were attached to a micro-connector and fixed to the skull with dental cement. Then, the wounds were closed. After surgery, each mouse was allowed to recover for 7 days.

A slip-ring was designed to free the behavioural movement of the mice for EEG and EMG recordings. The mice were adapted to the recording cable for 24 h before polygraphic recordings were obtained. Then, mice were continuously orally administered drugs at 7:00 PM for 14 days. After 14 days, the sleep-wakefulness states were monitored for a 24-h period.

### ***Vigilance state analysis***

Cortical EEG and EMG signals were amplified and filtered (EEG, 0.5-30 Hz; EMG, 20-200 Hz). They were then digitised at a sampling rate of 128 Hz and recorded using SleepSign software (Kissei Comtec, Nagano, Japan). Then, the vigilant states were automatically classified off-line by 4-s epochs into 3 stages, including wakefulness, REM sleep, and NREM sleep according to standard criteria (MM Yan et al 2011). Wakefulness is defined by a low-amplitude, high-frequency EEG recording with high EMG activity. REM sleep is characterised by a low-amplitude, high-frequency EEG recording associated with the absence of EMG activity, and the presence of EEG theta-activity (6-9Hz) in the recording can also be used to confirm this state. NREM sleep is commonly defined by a high-amplitude EEG

recording associated with a low-voltage EMG recording. The presence of high EEG delta activity (0.65-4 Hz) can also be used to characterise this state. Finally, defined sleep-wake stages were visually examined and corrected if necessary.

#### UPLC/Q-TOF-MS analysis

The UPLC/Q-TOF-MS analysis was carried out by a Waters Acquity Ultra Performance LC system (UPLC, Waters, USA) equipped with Q-TOF analyzer in a SYNAPT G1 system (Waters Corporation). The UPLC consisted of a vacuum degasser, a binary pump, an autosampler, a column heater and a diode array detector (DAD). The chromatographic separation was performed at 40°C on ACQUITY UPLC HESS C18 column (2.1 mm × 100 mm, 1.8 μm). The mobile phase consisted of acetonitrile (A) and water (B). The gradient program was used as follows: initial 0–11.5min, linear change from A:B (5:95, v/v) to A:B (95: 5, v/v); 11.5-13.5min holding for A:B (95: 5, v/v) in the positive ionization mode and initial 0-17min, linear change from A:B (5:95, v/v) to A:B (95: 5, v/v); and 17-19min holding for A:B (95: 5, v/v) in the negative ionisation mode. The flow rate was 0.4 ml/min, and the sample injection volume was 2 μL.

For Q-TOF-MS, Ultra-high pure helium (He) was used as the collision gas and high purity nitrogen (N<sub>2</sub>) as the nebulising gas. MS analysis was simultaneously performed using electrospray ionisation with positive and negative ionisation modes (ESI+ and ESI-) under the following optimised parameters: cone gas volume flow was 50 L/h; cone voltage was 40V; capillary voltage was 3kV and 2.5kV respectively (ESI+ and ESI-); ion source temperature was 100°C; desolvation gas rate was 800 L/h; mother ion collision energy was 6 eV; scan time was 0.2 s; and quality of the scanning range was Da 50~1000. All the acquisition and analysis of data were performed with Waters Mass Lynx V4.1 software.

#### *Statistical analysis*

All the data are expressed as the  $\bar{x} \pm S$ . Locomotor activity test data were analysed by repeated measures analysis of variance (ANOVA), and sleep recording data were analysed by one-way ANOVA using SPSS version 19.0. The least significant difference (LSD) method was used for comparisons among groups. In all cases, P values lower than 0.05 were considered statistically significant.

## Tables

Table S1 Impacts of time and group on loco-motor activity in mice

	<b>F</b>	<b>P</b>
<b>Time</b>	3.312	0.08
<b>Group</b>	5.220	0.08
<b>Time*Group</b>	6.755	0.00

Table S2 Average loco-motor activity numbers of different groups (Counts)

	<b>DZP</b>	<b>PTP1</b>	<b>PTP2</b>	<b>PTP3</b>	<b>SWFI</b>
$\bar{x}$	40433.07	51073.89	44241.61	53952.04	66863.91
<b>s</b>	11032.5	11856.12	10035.47	16030.42	11141.84
<b>SWFI(p)</b>	0.01*	0.025*	0.003*	0.59	

\* P value stands for comparison to SWFI.

Table S3: Compounds identified in PTP by high performance liquid chromatography-electrospray ionization/mass

N O	Rt(min)	[M+H]/[M- H](m/z)	Relative abundance <sup>a</sup> (%)	Formula	Identification	Component type
1	/0.52	/180.064	2.875102	C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	L-Tyrosine	Alkaloids
2	/0.52	/149.0506	1.396811	C <sub>9</sub> H <sub>10</sub> O <sub>2</sub>	E-p-coumaryl alcohol	Phenylpropanoi ds
3	/0.58	/341.1116	23.36166	C <sub>16</sub> H <sub>22</sub> O <sub>8</sub>	Coniferin	Phenylpropanoi ds
4	0.71	284.0995/	10.19231	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>5</sub>	Guanosine	Alkaloids
5	0.71	269.0886/	8.365385	C <sub>10</sub> H <sub>12</sub> N <sub>4</sub> O <sub>5</sub>	Inosine	Alkaloids
6	0.71	244.0933/	3.173077	C <sub>9</sub> H <sub>13</sub> N <sub>3</sub> O <sub>5</sub>	Cytidine	Alkaloids
7	0.71	136.0623/	10.76923	C <sub>5</sub> H <sub>5</sub> N <sub>5</sub>	Pedatisectine B	Alkaloids

8	1.30	268.1046/	37.11538	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	Adenosine	Alkaloids
9	/1.73	/137.0239	0.349203	C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>	Protocatechuic aldehyde	others
10	/3.20	/121.0501	1.524852	C <sub>4</sub> H <sub>10</sub> O <sub>4</sub>	Erythritol	others
11	3.22	279.1596/	7.980769	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	Dibutyl phthalate	Volatile oils
12	3.36	696.5414/	4.519231	C <sub>40</sub> H <sub>73</sub> NO <sub>8</sub>	1-O-glucosyl-N- 2'-palmitoyl-4,8 -sphingodienine	Cerebrosides
13	/4.68	/151.0395	0.88464	C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>	Anisic acid	Volatile oils
14	3.96 /5.02	255.0657/ /253.0501	13.84615 0.651845	C <sub>15</sub> H <sub>10</sub> O <sub>4</sub>	Chrysophanol	Anthraquinones
15	5.95 /0.58	343.1240/ /341.1084	4.038462 23.28018	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>	Sucrose	saccharides
16	/7.10	/329.2328	12.99034	C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>	Pinellic acid	Fatty acids
17	/14.01	/293.1753	32.68537	C <sub>17</sub> H <sub>26</sub> O <sub>4</sub>	Gingerol	others

Note:a : Relative abundance value is obtained by normalization method.

## Figures

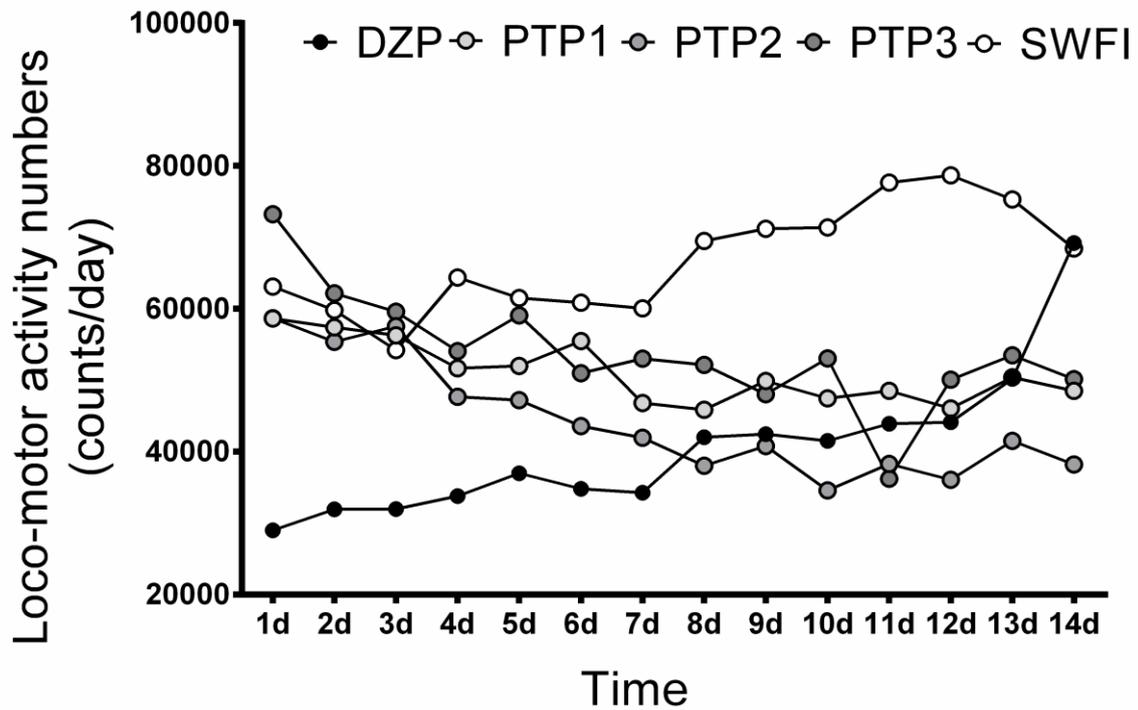


Figure S1 Locomotor activity numbers after intragastric administration over time. White, light Grey, middle Grey, dark Grey and black bars show the profiles for the placebo control group, PTP 1, PTP 2, PTP3 and positive control group, respectively.

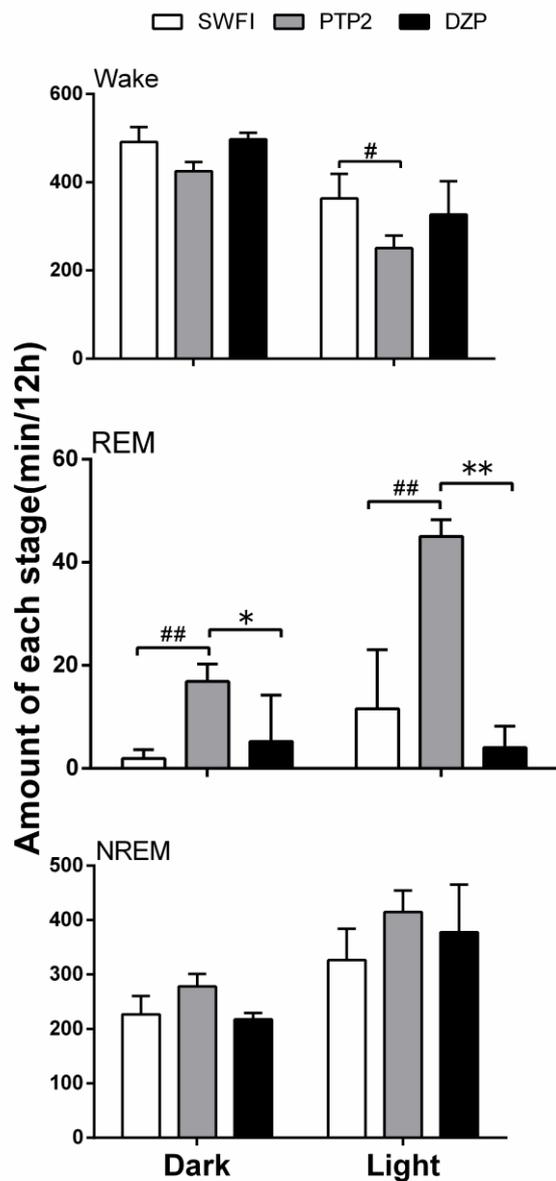


Figure S2 Changes in wakefulness, REM and NREM sleep over time. Each circle represents the hourly mean amount of each stage (A). Total time spent in different stage of sleep during the 12 h light and 12 h dark phases (B). White, Grey and black bars show the profiles for the placebo control group, PTP and positive control group, respectively. Values are  $\bar{x} \pm S$  (n = 6); #P<0.05 and ##P<0.01 indicate significant differences compared between PTP and SWFI, \*P<0.05 and \*\*P<0.01 indicate significant differences compared between PTP and DZP,  $\Delta$ P<0.05 and  $\Delta\Delta$ P<0.01 indicate significant differences compared between SWFI and DZP as assessed by ANOVA followed by the LSD test.

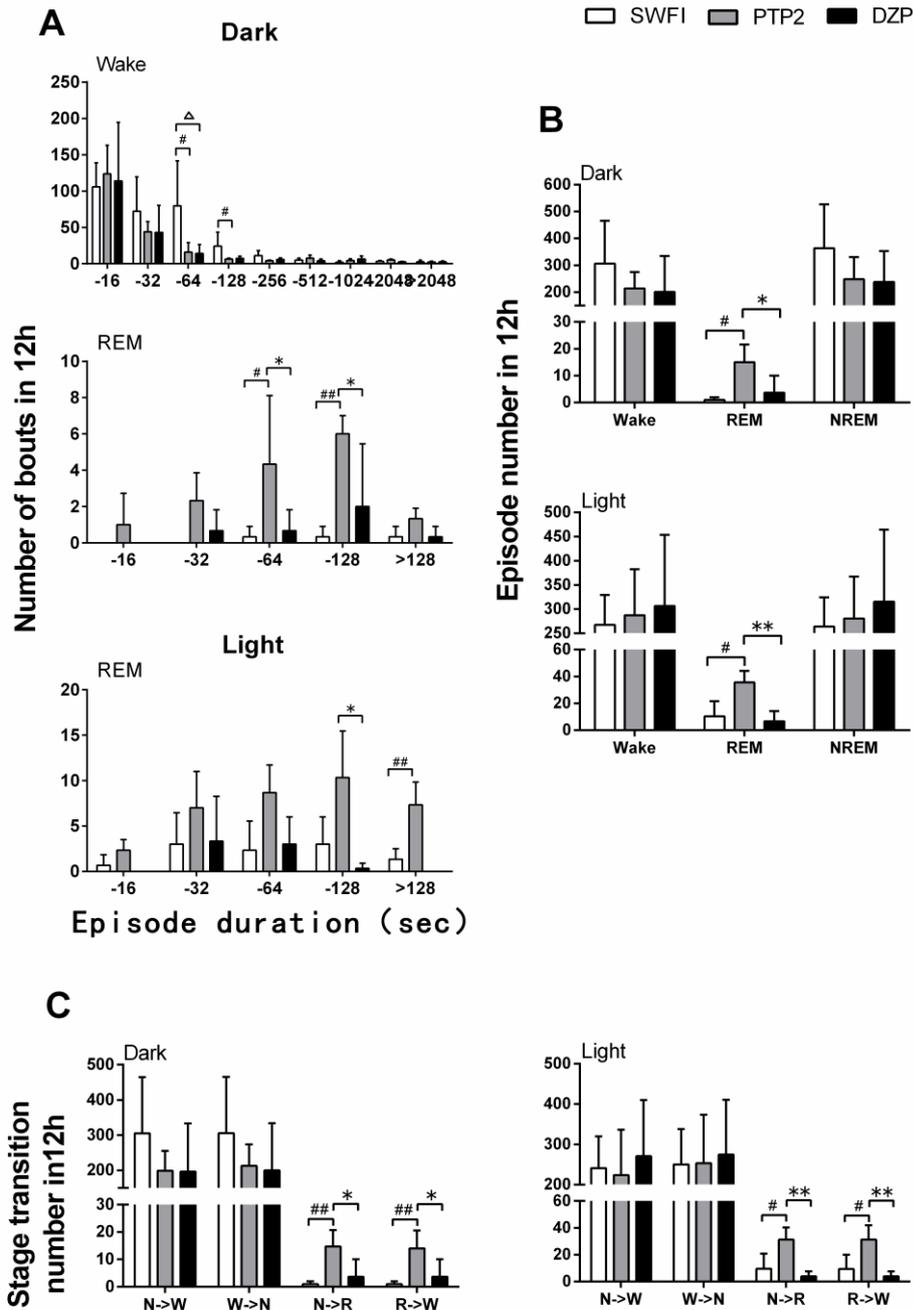


Figure S3 Characteristics of sleep-wake episodes. (A) Numbers of wakefulness and REM sleep bouts during dark and light. (B) Episode numbers in 12 h. (C) Sleep-wake state transitions during 12 h. White, Grey and black bars show the profiles for the placebo control group, PTP and positive control group, respectively. Values are  $\bar{x} \pm S$  ( $n = 6$ ); # $P < 0.05$  and ## $P < 0.01$  indicate significant differences compared between PTP and SWFI, \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant differences compared between PTP and DZP,  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  indicate significant differences compared between SWFI and DZP as assessed by ANOVA followed by the LSD test.

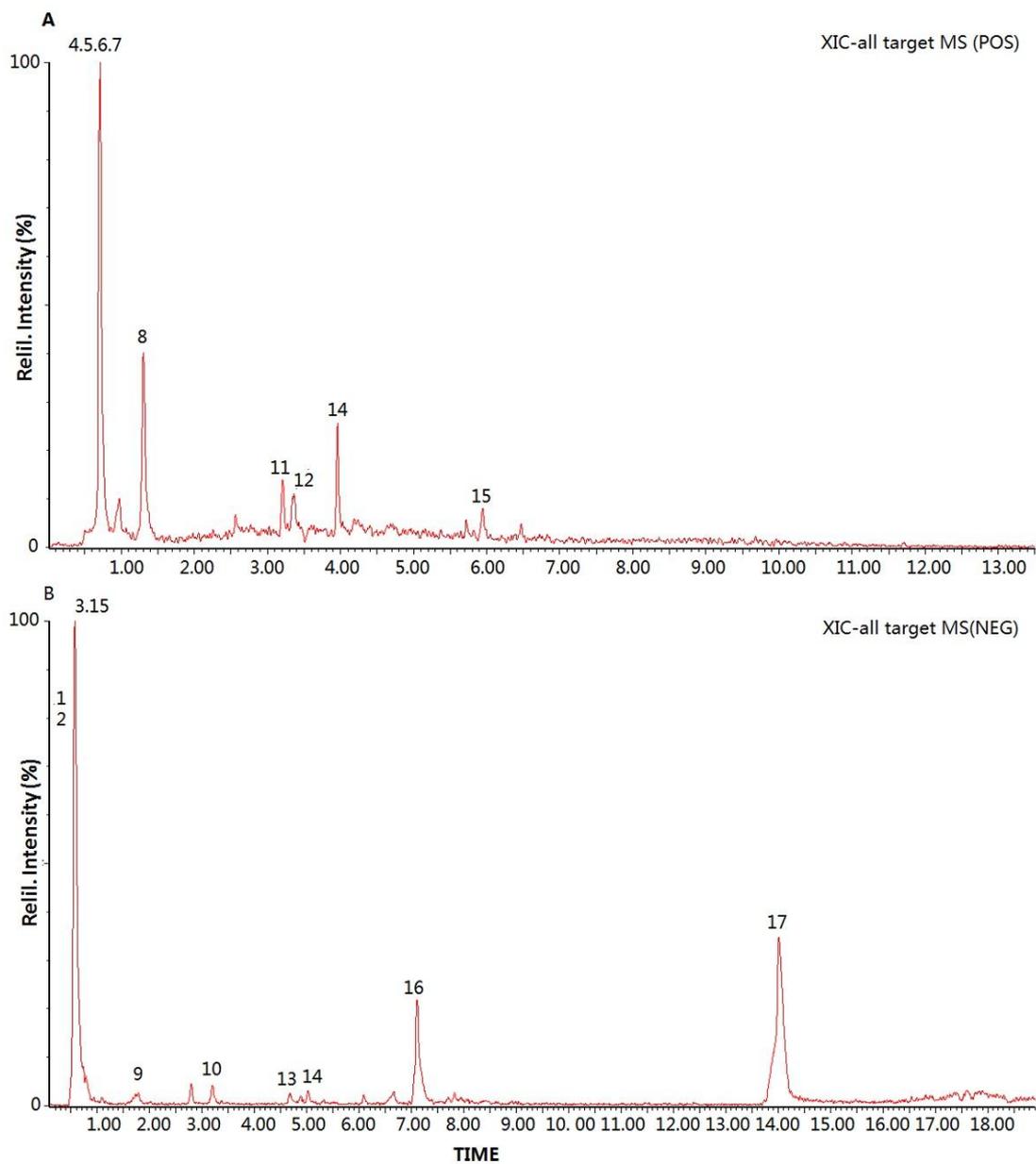


Figure S4 High-performance liquid chromatography-electrospray ionization/mass spectrometer ion chromatograms for PTP. (A) ES+TOF MS spectrum, (B) ES- TOF MS spectrum.