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(54) **Title:** APPLICATION OF COMPOSITION OF D-MANNURONIC DIACID IN TREATMENT OF PARKINSON'S DISEASE

(54) **发明名称:** 甘露糖醛二酸的组合物在治疗帕金森氏症中的应用

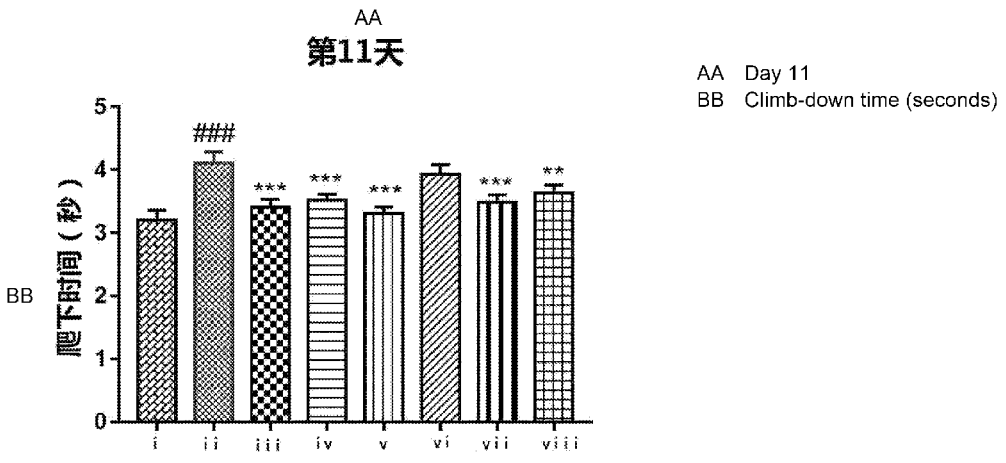

图 4

(57) **Abstract:** The present invention relates to an application of an oligosaccharide composition of D-mannuronic diacid in treatment of Parkinson's disease.

(57) **摘要:** 本发明涉及一种甘露糖醛二酸寡糖组合在治疗帕金森氏症方面的应用。

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DESCRIPTION

USE OF MANNURONIC DIACID COMPOSITION IN TREATMENT OF PARKINSON'S DISEASE

TECHNICAL FIELD

The present invention relates to the use of an optimal composition of mannuronic diacids obtained by a biological activity evaluation method in the treatment of Parkinson's disease.

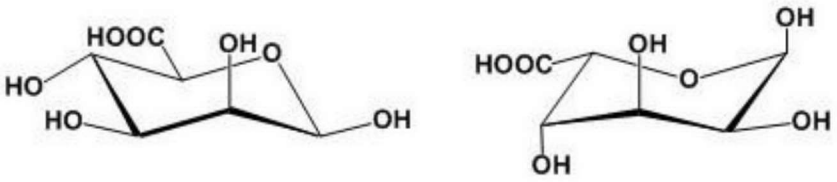
BACKGROUND OF THE INVENTION

Parkinson's disease (PD) is a common neurodegenerative disease occurring in the middle-aged and elderly people over 50-60 years old. The main lesions are located in the nigra and striatum pathways, leading to resting tremor, hypertonia and bradykinesia. Parkinson's disease is the fourth most common neurodegenerative disease among the elderly people, with 1% of people over 65 years old suffering from this disease and 0.4% of people over 40 years old suffering from this disease. The drugs for clinical treatment of Parkinson's disease are mainly compound levodopa formulation, dopamine receptor agonist, monoamine oxidase inhibitor, anticholinergic formulation and amantadine, etc., but there are some disadvantages such as large side effects and decreased long-term application effects.

Mannuronic diacids have been paid extensive attention due to their potential medicinal values. Mannuronic diacids are usually prepared by multiple steps with alginic acid as a raw material.

The polysaccharide molecule of the raw material, alginic acid, comprises an M segment formed of D-mannuronic acids linked by β-1,4-glucosidic bonds, a G segment formed of L-guluronic acids linked by α-1,4-glucosidic bonds, and an MG segment formed by hybridization of the two sacchorides. The structural

formulae of D-mannuronic acid and L-guluronic acid are shown in the following Formula (I):



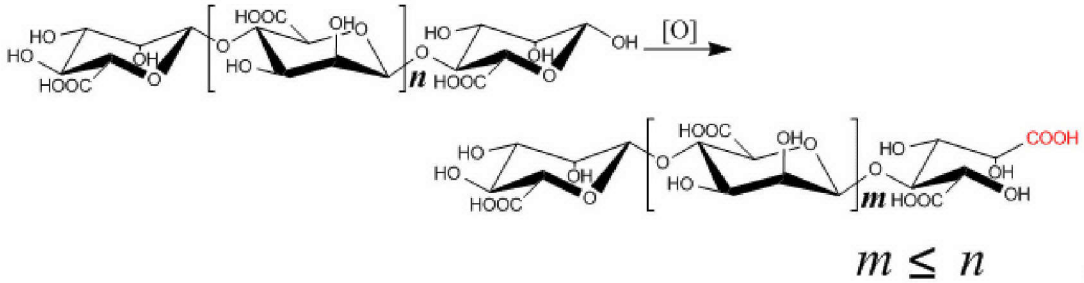
M; β-D-mannuronic acid G; α-L- guluronic acid (I)

The M segment and the G segment can be separated from the raw material, alginic acids. A common method can be simply described below: alginic acid is preliminarily degraded to give mixed polysaccharides of polymannuronic acid and polyguluronic acid; then the mixed polysaccharides are subjected to acidic precipitation to remove the polyguluronic acid therein, and further refinement is conducted to obtain a homopolymannuronic acid with a purity of more than 90% (hereinafter also referred to as "M-segment intermediate"). See, e.g., the methods disclosed in Chinese Patent Application No. 98806637.8 and CN02823707.2.

A common preparation method of oligomeric mannuronic acid is as follows: the M-segment intermediate obtained above is subjected to further acidolysis by heating under an acidic condition to obtain a small fragment mannuronic acid polymer having a desired molecular weight range. In addition, the degradation efficiency can be improved by an oxidative degradation method; meanwhile, the reducing end can be oxidized to a ring-opened saccharic diacid, see Chinese Patent Application No. 200580009396.5 (Patent literature 1) filed by Meiyu Geng, *et al.* and US Patent No. 8,835,403 B2 (Patent literature 2) for details. For convenience of description, Patent literatures 1 and 2 are hereinafter collectively referred to as prior documents, of which are incorporated herein by reference in their entirety.

The reaction to obtain of mannuronic diacid disclosed in prior documents can be represented by the following reaction equation (II), that is, the aldehyde

group at position C1 of mannuronic acid at the reducing end of oligomannuronic acid polysaccharide is oxidized to carboxyl group.



$$m \leq n \qquad \text{(II)}$$

In the above oxidative conversion process, a commonly used oxidant is an alkaline copper sulfate solution, i.e. Fehling's reagent. Prior documents adopt this oxidation method. Specifically, under an alkaline condition, the reaction substrate polymannuronic acid, i.e. the above M-segment intermediate, is added to a copper sulfate solution and reacted in a boiling water bath for 15 minutes to 2 hours. This method uses Cu^{2+} ion as an oxidant to oxidize the aldehyde group, and a brick-red cuprous oxide precipitate is generated in the reaction. This reaction is often used to identify a reducing sugar.

Prior documents disclose that oligomannaric acids have effects against Alzheimer's disease (AD) and Diabetes Mellitus. The pathogenesis of Alzheimer's disease and type 2 diabetes is closely related to amyloids (β-amyloid and amylin). Amyloid protein aggregates and then produces protein oligomers, which further aggregate to form fibers. These protein aggregates are cytotoxic, induces an oxidative reaction in cells to damage mitochondria, and triggers a cascade reaction such as inflammatory reaction, causing damages to a large number of neurons and β cells, and ultimately leading to onset of Alzheimer's disease and type 2 diabetes. Oligomannaric acids target amyloid protein and antagonize the cascade reactions induced by the amyloid protein, and therefore have the effects of preventing and treating Alzheimer's disease and type 2 diabetes.

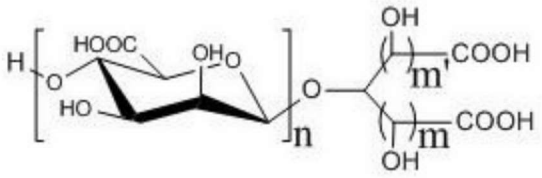
The prior document CN106344592A discloses the application of mannuronic acid oligosaccharide and its derivatives with carboxyl at position 1

of the reducing end in the treatment of Parkinson's disease, and also discloses the pharmacodynamic activity of tetrasaccharide-to-decasaccharide mixture in the treatment of Parkinson's disease.

SUMMARY OF THE INVENTION

The invention relates to the use of a mannuronic diacid oligosaccharide composition in the treatment of Parkinson's disease. The present invention also relates to a method for treating Parkinson's disease, which comprises administering a therapeutically effective amount of the mannuronic diacid oligosaccharide composition of the present invention to a patient in need thereof.

The mannuronic diacid oligosaccharide composition disclosed by the present invention comprises mannuronic diacids having Formula (III) or a pharmaceutically acceptable salt thereof:



Formula (III),

wherein n is an integer selected from 1 to 9, m is selected from 0, 1 or 2, m' is selected from 0 or 1, and

wherein,

the total weight of mannuronic diacids wherein n=1-5 accounts for no less than 60% of the total weight of the composition;

the total weight of mannuronic diacids wherein n=1-2 accounts for less than 60% of the total weight of the composition.

Applicants have found that the mannuronic diacid composition having a specific consitution has a favorable effect on the treatment of Parkinson's disease, while reducing the production cost. Also, it is beneficial to improve the quality of life of patients due to the safety derived from natural products.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows mass spectra of disaccharide, trisaccharide and tetrasaccharide in product A.

Figure 2 shows mass spectra of pentasaccharide, hexasaccharide and heptasaccharide in product A.

Figure 3 shows mass spectra of octasaccharide, nonasaccharide and decasaccharide in product A.

Figure 4 shows the effects of different oligosaccharide compositions and hexasaccharides on the crawling-down time of PD animals on the 11th day. The samples corresponding to the numbers on the abscissa in the Figure are: i: control group; ii: model group; iii: product A; iv: product B; v: product C; vi: product D; vii: comparative experimental sample; viii: hexasaccharide.

Figure 5 shows the effects of different oligosaccharide compositions and hexasaccharides on the latency of PD animals on the 11th day; wherein the symbols on the abscissa are the same as those in Figure 4.

Figure 6 shows the effects of different oligosaccharide compositions and hexasaccharides on the crawling-down time of PD animals on the 14th day; wherein the symbols on the abscissa are the same as those in Figure 4.

Figure 7 shows the effects of different oligosaccharide compositions and hexasaccharides on the latency of PD animals on the 14th day; wherein the symbols on the abscissa are the same as those in Figure 4.

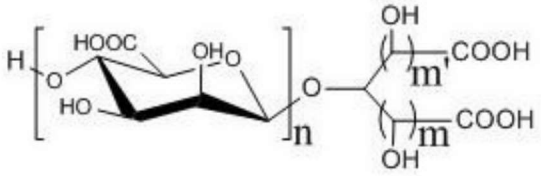
Figure 8 shows the effects of different oligosaccharide compositions and hexasaccharides on the crawling-down time of PD animals on the 17th day; wherein the symbols on the abscissa are the same as those in Figure 4.

Figure 9 shows the effects of different oligosaccharide compositions and hexasaccharides on the latency of PD animals on the 17th day; wherein the symbols on the abscissa are the same as those in Figure 4.

DETAILED DESCRIPTION OF THE INVENTION

Various aspects of the present invention will be described in detail below, but the present invention is not limited to these specific embodiments. Those skilled in the art can make some modifications and adjustments to the present invention according to the the substantial disclosure below, and these adjustments are also within the scope of the present invention.

The present invention relates to the use of the mannuronic diacid oligosaccharide composition in the treatment of Parkinson's disease. The mannuronic diacid oligosaccharide composition used comprises mannuronic diacids having Formula (III) or a pharmaceutically acceptable salt thereof:



Formula (III),

wherein n is an integer selected from 1 to 9, m is selected from 0, 1 or 2, m' is selected from 0 or 1, and

wherein,

the total weight of mannuronic diacids wherein n=1-5 accounts for no less than 60% of the total weight of the composition;

the total weight of mannuronic diacids wherein n=1-2 accounts for less than 60% of the total weight of the composition.

The mannuronic diacid oligosaccharide composition of the present invention is a mixture of mannuronic diacids with different polymerization degrees, and the main components thereof are mannuronic diacid oligosaccharides with a polymerization degree of 2 to 10. The most active saccharides in mannuronic diacids are tetrasaccharide to decasaccharide, especially hexasaccharide. However, unlike the known prior art, the inventors have found that adding a certain proportion of less active disaccharide and trisaccharide to the most active tetrasaccharide to decasaccharide does not reduce the biological activity and even increases the activity under the same administration dosage in mass. Without being bound by any theory, it is

speculated that this may be due to the synergistic effect of the small molecular weight disaccharide and trisaccharide when mixed with other oligosaccharides although they cannot work alone. However, when the proportion of disaccharide and trisaccharide is too high, the overall activity of the composition would be reduced due to the low activity of disaccharide and trisaccharide *per se*. Therefore, the proportion of disaccharide and trisaccharide in the composition must be controlled within a certain range.

In the actual preparation process, a certain amount of disaccharide and trisaccharide will be produced in the oxidative degradation reaction, and usually will be removed from the product after separation in order to avoid affecting the pharmaceutical effect of the product due to its low activity. However, based on the above findings of the inventors, it is not required to separate and remove disaccharide and trisaccharide in the oxidative degradation products, and it is only required to control the conditions of the oxidative degradation reaction to control the proportion of disaccharide and trisaccharide within a certain range. The activity of the resulted composition can reach or even be better than that of the composition disclosed in the piror applications. Moreover, since disaccharide and trisaccharide are not considered as impurities to be removed, the product yield is also significantly higher than that disclosed in the prior applications. Thus, it greatly reduces the production cost, reduces the waste discharge, thereby being easier to realize in the actual production, and being easier to realize industrial large-scale production.

According to a preferred embodiment, in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacids with m+m'=1 or 2 is no less than 50% of the total weight of the composition, preferably 60%-90%, more preferably 70%-90%. In particular, in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacids with m+m'=1 is no less than 10% of the total weight of the composition, preferably 30-40%. In another preferred embodiment, in the mannuronic diacid oligosaccharide composition, the total weight of

mannuronic diacids with m+m'=2 is no less than 10% of the total weight of the composition, preferably 30-50%.

According to a preferred embodiment, in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacid oligosaccharide wherein n=1-5 accounts for 80-95% of the total weight of the composition.

According to a preferred embodiment, in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacid oligosaccharide wherein n=1-2 accounts for 10-50% of the total weight of the composition, preferably 25-50%.

According to a preferred embodiment, in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacid oligosaccharide wherein n=1-3 accounts for 20-70% of the total weight of the composition.

According to a preferred embodiment, in the mannuronic diacid oligosaccharide composition, the proportion of the total weight of mannuronic diacids wherein n=1-3 to the total weight of mannuronic diacids wherein n=4-7 is between 1.0 and 3.5, preferably between 1.0 and 3.0.

According to a preferred embodiment, the weight percentage content of mannuronic diacids with each of the polymerization degrees in the mannuronic diacid oligosaccharide composition is: disaccharide 5-25%, trisaccharide 15-30%, tetrasaccharide 15-28%, pentasaccharide 5-25%, hexasaccharide 2-20%, heptsaccharide 2-20%, octasaccharide 2-20%, nonasaccharide 2-20%, decasaccharide 2-20%. In particular, the weight percentage content of oligosaccharides in the composition is: disaccharide 5-25%, trisaccharide 15-30%, tetrasaccharide 15-28%, pentasaccharide 10-20%, hexasaccharide 5-15%, heptsaccharide 3-10%, octasaccharide 2-5%, nonasaccharide 1-5%, decasaccharide 1-5%. More preferably, the weight percentage content of oligosaccharides in the composition is: disaccharide 10-20%, trisaccharide 18-30%, tetrasaccharide 15-28%, pentasaccharide 15-20%, hexasaccharide

5-10%, heptsaccharide 3-5%, octasaccharide 2-5%, nonaccharide 1-3%, decasaccharide 1-3%.

In the mannuronic diacid oligosaccharide composition of the present invention, the pharmaceutically acceptable salt thereof can be sodium salt or potassium salt.

The inventors of the present patent application have found that, when the oligosaccharides of different polymerization degree are complexed according to certain proportions, a high-activity oligosaccharide composition can be obtained, of which the activity is higher than that of the most active hexasaccharide. In particular, the composition added with a specific proportion of disaccharide and trisaccharide has higher activity than the composition without disaccharide and trisaccharide. The proportion of each oligosaccharide in the high-activity oligosaccharide composition can have the following proportion:

The total weight of mannuronic diacids wherein n=1-5 in the composition accounts for no less than 60%of the total weight of the composition, preferably 80-95%. The total weight of mannuronic diacids wherein n=1-2 accounts for less than 60% of the total weight of the composition, preferably 10-50%, more preferably 25-50%. The total weight of mannuronic diacid oligosaccharide wherein n=1-3 accounts for 20-70% of the total weight of the composition. The ratio of the total weight of the mannuronic diacid oligosaccharide wherein n=1-3 to the total weight of the mannuronic diacid oligosaccharide wherein n=4-7 is between 1.0 and 3.5, preferably between 1.0 and 3.0.

The medicament for the treatment of Parkinson'disease of the present invention comprises a mannuronic diacid oligosaccharide composition, which comprises mannuronic diacids having Formula (III) or pharmaceutically acceptable salts thereof, and one or more pharmaceutically acceptable carriers. The medicament of the present invention can be in the form of tablets, hard capsules, soft capsules, enteric capsules, microcapsules, granules, syrups,

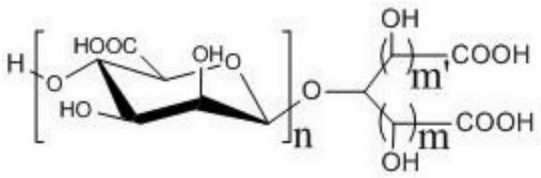
injections, granules, emulsions, suspensions, solutions and sustained-release formulation for oral or non-oral administration.

The pharmaceutically acceptable carrier of the present invention refers to a pharmaceutically acceptable carrier known to those skilled in the art. The pharmaceutically acceptable carrier of the present invention includes, but is not limited to, fillers, wetting agents, binders, disintegrants, lubricants, adhesive, glidants, taste masking agents, surfactants, preservatives, etc. Fillers include, but are not limited to lactose, microcrystalline cellulose, starch, saccharide powder, dextrin, mannitol, calcium sulfate, etc. Wetting agents and binders include, but are not limited to sodium carboxymethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, gelatin, sucrose, polyvinylpyrrolidone, etc. Disintegrants include, but are not limited to sodium carboxymethyl starch, crosslinked polyvinylpyrrolidone, crosslinked sodium carboxymethyl cellulose, low substituted hydroxypropyl cellulose, etc. Lubricants include, but are not limited to, magnesium stearate, silica gel micropowder, talc, hydrogenated vegetable oil, polyethylene glycol, magnesium lauryl sulfate, etc. Adhesive includes, but are not limited to, Arabic gum, alginic acid, calcium carboxymethylcellulose, sodium carboxymethylcellulose, glucose binders, dextrins, dextrose, ethyl cellulose, gelatin, liquid glucose, guar gum, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, magnesium aluminum silicate, maltodextrin, methylcellulose, polymethacrylates, polyvinylpyrrolidone, pregelatinized starch, sodium alginate, sorbitol, starch, syrup, and tragacanth gum. Glidants include, but are not limited to colloidal silica, powdered cellulose, magnesium trisilicate, silica and talc. Taste masking agents include, but are not limited to, aspartame, stevioside, fructose, glucose, syrup, honey, xylitol, mannitol, lactose, sorbitol, maltitol, and glycyrrhizin. Surfactants include, but are not limited to Tween -80 and poloxamer. Preservatives include, but are not limited to, parabens, sodium benzoate, potassium sorbate, etc.

As used herein, the term “treatment” generally refers to achieving a desired pharmacological and/or physiological effect. This effect can be preventive according to the complete or partial prevention of the disease or its symptoms; and/or can be therapeutic according to partial or complete stabilization or cure of the disease and/or side effects due to the disease. As used herein, “treatment” covers any treatment of a patient’s disease, including: (a) prevention of diseases or symptoms occurring in patients who are susceptible to disease or symptoms but have not yet been diagnosed with the disease; (b) inhibiting the symptoms of the disease, i.e. preventing its development; or (c) relieving the symptoms of the disease, i.e. causing the disease or the deterioration of the symptoms.

Mannuronic diacid oligosaccharide composition

The mannuronic diacid oligosaccharide composition for the treatment of Parkinson’s disease of the present invention comprises mannuronic diacids having Formula (III) or a pharmaceutically acceptable salt thereof:



Formula (III),

wherein n is an integer selected from 1 to 9, m is selected from 0, 1 or 2, m’ is selected from 0 or 1, and

wherein,

the total weight of mannuronic diacids wherein n=1-5 accounts for no less than 60% of the total weight of the composition;

the total weight of mannuronic diacids wherein n=1-2 accounts for less than 60% of the total weight of the composition.

In an exemplary embodiment, the preparation method of the mannuronic diacid oligosaccharide composition for the treatment of Parkinson’s disease comprises the following steps:

(1) Preparation of the mannuronic diacids products:

Preparation of M segment intermediate. As described above, the raw material M-segment intermediate used in the present invention can be prepared by a method known in the prior art, e.g., the methods disclosed in Chinese Patent Application No. 98806637.8 and CN02823707.2. A common method can be simply described below: alginic acid is preliminarily degraded to give mixed polysaccharides of polymannuronic acid and polyguluronic acid; then the mixed polysaccharides are subjected to acidic precipitation to remove the polyguluronic acid therein, and further refinement is conducted to obtain a homopolymannuronic acid with a purity of more than 90%, i.e., an M-segment intermediate.

Ozone oxidative degradation. The M-segment intermediate is dissolved in an appropriate amount of water and stirred at room temperature or under heating condition. With continuous introduction of ozone, the reaction starts. The pH value of the reaction can be adjusted to 3-13, preferably 4-10, more preferably 6-8 by dropwise adding dilute hydrochloric acid or dilute NaOH solution. The temperature is preferably 0-70 °C, more preferably 10-45 °C. After the completion of the reaction, the introduction of ozone is stopped and the pH is adjusted to neutral.

Membrane separation and purification. The reaction product obtained above is formulated into a solution at a concentration of about 10% and separated by a molecular cut-off membrane to remove degradation products below monosaccharide. The retentate is collected. The MWCO of the molecular cut-off membrane used is 1000 Da - 3000 Da, preferably 2000 Da. The collected liquid is concentrated on a rotary evaporator and dried under vacuum to obtain an oligomannuronic diacid mixture. After analysis, it is found that these products are all compositions of oligosaccharide from disaccharide to decasaccharide with contents being within certain proportion ranges. Specific examples of this preparation method can be found in examples 1-3, in which

three compositions A, B and C were respectively prepared according to the aforementioned methods.

(2) Preparation of oligosaccharides with a single polymerization degree

The oligosaccharide mixture obtained in step (1) is dissolved to a concentration of about 10%, separated on a P6 gel chromatographic column, and subjected to ultraviolet detection to collect each effluent component. The components having the same polymerization degree are combined. Nine components of disaccharide to decasaccharide were collected, desalted by G10 gel column chromatography, concentrated by rotary evaporator, and dried in vacuum. A specific purification and preparation process is shown in example 4. The operations such as column chromatography, desalting and drying are known to those skilled in the art.

(3) Activity comparison of oligosaccharide compositions

The prepared composition is compared with the hexasaccharide purified in step (2) for the pharmacological activity. The results show that the oligosaccharide composition of the present invention is significantly better than the most active hexasaccharide in the oligosaccharides with single polymerization degree, while the activity of the composition without disaccharide and trisaccharide is slightly lower than that of hexasaccharide. Accordingly, it can be seen that the oligosaccharides with different polymerization degrees can play a synergistic effect after being combined. When the proportion of disaccharide to hexasaccharide in the composition is no less than 60%, and the proportion of disaccharide and trisaccharide is less than 60%, the activity of the composition is the highest. However, when the proportion of disaccharide and trisaccharide is more than 60%, the activity of the composition would also decrease.

Animal model and steps for evaluating pharmacodynamic activity

Animal model for anti-Parkinson's Disease (PD) pharmacodynamic evaluation

Mice were randomly divided into 8 groups: blank control group, MPTP model group and dosing group, with 14 animals in each group. Animals were divided into groups and given drugs on the same day. The blank control group and the MPTP model group were given saline solution by intragastric administration, and the other groups were given corresponding drugs once a day for 17 consecutive days. Modeling drugs were given from the 6th day. The animals in the blank control group were given 10 ml/kg of saline subcutaneously, and the other animals were given 25 mg/kg of MPTP subcutaneously once a day for five days.

Behavioral tests were carried out on the 11th, 14th and 17th days respectively. The mouse head was gently place upward on the rough top of the rod (diameter of 8 mm, height of 55 cm). The adjustment time of mice from head-up to head-down is the latency (T-turn), and the time of mice from downward movement to all limbs reaching the bottom of the rod is recorded as the climbing-down time (T-LA). More than 30 seconds would be recorded as 30 seconds. Each mouse was tested 5 times and the results were averaged.

MPTP has selective destructive effect on dopaminergic neurons in substantia nigra. MPTP-induced PD animal model is the most classical animal model similar to pathological changes and clinical characteristics of human Parkinson's disease. The main symptoms of PD are resting tremor, increased muscle tension, decreased movement, etc. The turning time and climbing-down time of rod climbing experiment can represent the overall activity and coordination ability of mice.

Advantages of the present invention are further illustrated in the following nonlimiting examples. However, the specific materials and amounts thereof as well as other experimental conditions used in the examples should not be construed as limiting the present invention. Unless otherwise specified, the parts, proportions, percentages, and the like in the present invention are all calculated by mass.

EXAMPLE

Example 1:

Step 1): Preparation of a mannuronic diacid oligosaccharide mixture

An M-segment intermediate was prepared by the method disclosed in prior patents. The specific operations are briefly described below: 5 kg of sodium alginate was formulated into a solution of about 10%, and the pH was adjusted to about 3.0 by adding dilute hydrochloric acid. The solution was heated to 80 °C, and stirred. It was allowed to react for 10 hr before the heating was stopped. After cooling to room temperature, the pH was adjusted to 9.0 by adding NaOH, and further adjusted to 2.85 by adding dilute hydrochloric acid. The solution was centrifuged at 5000 rpm for 10 min. The supernatant was collected, and adjusted to pH 1.0 by adding HCl. After centrifugation, the precipitate was collected, concentrated on a rotary evaporator, and dried under vaccum to give 1500 g of the M-segment intermediate. 500 g of the M-segment intermediate was weighed, and dissolved in distilled water to prepare a solution in a volume of 5 L. The solution was adjusted to pH 6.5 with NaOH, and heated in a water bath to control the reaction temperature at 75 °C. The gas flow rate at the outlet of an oxygen cylinder and the power of an ozone generator were adjusted such that ozone was fed into the reaction solution at a mass concentration flow rate of 8 g/hr. After 4 hr of reaction, the feeding of ozone was stopped, and a suitable amount of water was added to adjust the concentration of the solution to about 10%. The solution was filtered through an ultrafiltration membrane with a molecular weight cut-off of 2,000 Da to collect a retentate. The collected liquid was concentrated on a rotary evaporator and dried under vacuum to obtain 350 g of mannuronic diacid product A.

Step 2): Analysis of proportions and structures of oligosaccharides with various polymerization degrees in mannuronic diacid product A

100 mg of the above dried mannuronic diacid product A was accurately weighed, dissolved in water to a concentration of 10 mg/mL, and passed through a 0.22 μm filter membrane to obtain a test sample solution. The

proportions of oligosaccharides with different polymerization degrees in the composition were determined by Superdex peptide molecular exclusion chromatography (GE Co.) in combination with multi-angle laser light scattering (MALS, Wyatt Co.). The experimental conditions were as follows:

Chromatographic column: Superdex peptide 10/300G1

Mobile phase: 0.1 mol/L NaCl

Injection volume: 10 μL

Flow rate: 0.3 mL/min

Test results: from disaccharide to decasaccharide were represented by dp2-dp10, respectively, dp2 was 19%, dp3 was 25%, dp4 was 22%, dp5 was 13%, dp6 was 9%, dp7 was 6%, dp8 was 3%, dp9 was 2% and dp10 was 1%.

Step 3): LC-MS analysis of structures of oligosaccharides with various polymerization degrees in mannuronic diacid product A

Experimental conditions:

Chromatographic column: Superdex peptide 10/300G1

Mobile phase: 20% methanol + 80% 80 mmol/L NH_4Ac

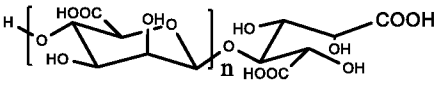
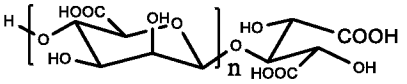
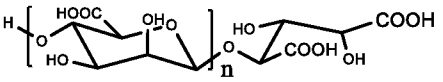
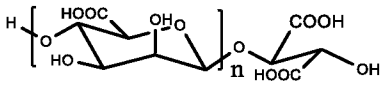
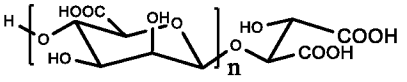
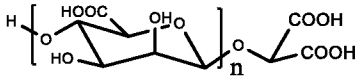
Flow rate: 0.1 mL/ min

Column temperature: 25°C±0.8 °C.

Mass spectrometry conditions: Agilent 6540 QTOF; ion source: ESI collision voltage 120 V; negative ion mode. The width of the acquired signal (m/z) was 100-1000.

The mass spectra of oligosaccharides with various polymerization degrees are shown in Figures 1-3. Various signal peaks in the mass spectra were assigned, confirming the molecular structure of all oligosaccharides in product A, i.e., the structure shown in General Formula (III). See Table 1 below for the signal assignments and the structures corresponding to the signals.

Table 1: six diacid structures in oligosaccharides with different polymerization degrees in product A and their mass-to-charge ratios in mass spectra

No.	Molecular structure	Molecular Formula	Mass-to-Charge Ratio (m/z)								
			n=1 $[M-1]^-$	n=2 $[M-1]^-$	n=3 $[M-1]^-$	n=4 $[M-1]^-$	n=5 $[M-1]^-$	n=6 $[M-1]^-$	n=7 $[M-2]^{2-}$	n=8 $[M-2]^{2-}$	n=9 $[M-2]^{2-}$
1		$(C_6H_8O_6)_nC_6H_{10}O_8$ n=1-9	385	561	737	913	1089	1265	720	808	896
2		$(C_6H_8O_6)_nC_5H_8O_7$ n=1-9	355	531	707	883	1059	1235	705	793	881
3		$(C_6H_8O_6)_nC_5H_8O_7$ n=1-9	355	531	707	883	1059	1235	705	793	881
4		$(C_6H_8O_6)_nC_4H_6O_6$ n=1-9	325	501	677	853	1029	1205	690	778	866
5		$(C_6H_8O_6)_nC_4H_6O_6$ n=1-9	325	501	677	853	1029	1205	690	778	866
6		$(C_6H_8O_6)_nC_3H_4O_5$ n=1-9	295	471	647	823	999	1175	675	763	851

It was found from the above mass spectrometric structural analysis that the mannuronic acid at the reducing end of the sugar chain in product A was oxidized to a saccharic diacid structure (see General Formula III for the structure), which could be a mannaric diacid structure comprising 6 carbon atoms (m+m'=3) with a content of about 10%~30%, or a decarboxylation product of mannaric diacid, i.e., a saccharic diacid comprising 5 carbons (m+m'=2) (30~50%) and a saccharide diacid with 4 carbons (m+m'=1) (30%~40%).

Example 2:

100 g of the M-segment intermediate in example 1 was weighed, and dissolved in distilled water to prepare a solution with a volume of 0.8 L. The solution was adjusted to pH 4.0 with NaOH, and the reaction was carried out at room temperature (25°C). The gas flow rate at the outlet of an oxygen cylinder and the power of an ozone generator were adjusted such that ozone was fed into the reaction solution at a mass concentration flow rate of 1 g/hr. After 10 hr of reaction, the feeding of ozone was stopped, and a suitable amount of water was added to adjust the concentration of the solution to about 15%. The solution was filtered through an ultrafiltration membrane with a molecular weight cut-off of 1,000 Da to collect a retentate. The collected liquid was concentrated on a rotary evaporator and dried under vacuum to obtain 80 g of mannuronic diacid product B.

The proportions of oligosaccharides components with various polymerization degrees in B were determined by Superdex peptide molecular exclusion chromatography (GE Co.) in combination with multi-angle laser light scattering (MALS, Wyatt Co.). The measurement method was the same as the relevant part in example 1. Test results: from disaccharide to decasaccharide were represented by dp2-dp10, respectively, dp2 was 20%, dp3 was 25%, dp4 was19%, dp5 was 12%, dp6 was 9%, dp7 was 5%, dp8 was 5%, dp9 was 3% and dp10 was 2%.

Example 3:

100 g of the M-segment intermediate of example 1 was weighed, dissolved in distilled water to prepare a solution with a volume of 1.5 L. The solution was adjusted to pH 9.0 with NaOH, and the reaction was carried out in a water bath at 45 °C. The gas flow rate at the outlet of an oxygen cylinder and the power of an ozone generator were adjusted such that ozone was fed into the reaction solution at a mass concentration flow rate of 3 g/hr. After 2 hr of reaction, the feeding of ozone was stopped, and a suitable amount of water was added to adjust the concentration of the solution to about 5%. The solution was filtered through an ultrafiltration membrane with a molecular weight cut-off of 3,000 Da to collect a retentate. The collected liquid was concentrated on a rotary evaporator and dried under vacuum to obtain 60 g of mannuronic diacid product C.

The proportions of oligosaccharides with various polymerization degrees in C were determined by Superdex peptide molecular exclusion chromatography (GE Co.) in combination with multi-angle laser light scattering (MALS, Wyatt Co.). The measurement method was the same as the relevant part in example 1. Test results: from disaccharide to decasaccharide were represented by dp2-dp10, respectively, dp2 was 8%, dp3 was 20%, dp4 was 28%, dp5 was 19%, dp6 was 13%, dp7 was 6%, dp8 was 3%, dp9 was 2%, and dp10 was 1%.

Example 4:

Preparation of mannuronic diacid oligosaccharide with single polymerization degree, which was as follows:

1. Sample preparation: 300 g of mannuronic diacid product A prepared in example 1 was weighed, dissolved in water, prepared into 1000 mL of concentrated solution, and placed in a refrigerator at 4 °C for use. For each use,

50 mL was taken out and was 1:2 diluted with water, and then suction filtered through a 0.22 μm ultrafiltration membrane.

2. Chromatographic separation conditions: The chromatograph was AKTA pure 150 (purchased from GE Co.) equipped with a UV detector and an automatic collector. Separation chromatographic column: 1.2 kg of BioGel P6 (purchased from Bio-Rad Co.) was mixed with deionized water, vacuum degassed, manually filled into a glass column (inner diameter: 10 cm), rinsed with 10 column volumes of pure water. The chromatographic column bed was stable and the height was 1.0 m. Then, the mobile phase was changed to a 0.02 M NaCl solution, and after equilibration with 10 column volumes, sample loading was initiated.

3. Sample loading and separation: The flow rate of the pump was set at 1 mL/min. After 100 mL of the sample solution was pumped to the top of the column through the chromatograph's own pump, it was switched to the mobile phase and eluted at a flow rate of 5 mL/min. After outflow of the dead water volume, automatic collection was initiated and 50 mL was collected per tube.

4. The sample loading was repeated, and after 20 repetitions of preparation, the same fractions were combined, concentrated on a rotary evaporator, and lyophilized to obtain a total of 9 oligosaccharides with single polymerization degree from disaccharide to decasaccharide.

Example 5

A pharmacological activity evaluation was conducted between the compositions and hexasaccharide to examine the synergistic effect of the oligosaccharides with different polymerization degrees in the compositions and the range of proportions of the oligosaccharides.

Sample preparation:

Composition product D:

The mannuronic diacid oligosaccharides with single polymerization degree as prepared in example 4 were accurately weighed from disaccharide to

decasaccharide by the polymerization degree. The weight of each saccharide taken out was as follows: 3.0 g of disaccharide, 3.0 g of trisaccharide, 1.5 g of tetrasaccharide, 1.5 g of pentasaccharide, 0.4 g of hexasaccharide, 0.2 g of heptasaccharide, 0.2 g of octasaccharide, 0.1 g of nonasaccharide, and 0.1 g of decasaccharide. They were uniformly mixed to obtain 10 g of composition product D.

Preparation of comparative experimental samples

A tetrasaccharide-to-decasaccharide containing mixture was prepared by referring to the methods disclosed in examples 1 and 2 of the prior document CN106344592A.

1g of sodium polymannuronate (weight average molecular weight 8235Da, provided by Shanghai Green Valley Pharmaceutical Co., Ltd.) was weighed and added with appropriate amount of distilled water to prepare 1% (weight percent) aqueous solution of sodium polymannuronate. The pH value of the 1% aqueous solution of sodium polymannuronate was adjusted to 4 with hydrochloric acid, and then the aqueous solution was placed in an autoclave. The reaction was subjected to heating at 110°C for 4 hours. The reacted solution was removed from the autoclave and allowed to cool. After cooling, the pH value of the reacted solution was adjusted with NaOH solution to obtain neutral liquid. Under the condition of stirring, the neutral liquid was slowly added into ethanol with a volume of 4 times the volume of the liquid. The alcohol precipitation was carried out, and the solution was left to stand overnight. The solid substance obtained by alcohol precipitation was filtered and separated, and the absolute ethanol was used to wash the solid substance obtained from filtering and separation during the filtering and separation process. Finally a white filter cake was produced. The filter cake was filtered in an oven at 60°C to obtain crude alginate oligosaccharide.

5g of crude alginate oligosaccharide was prepared into a 5% (weight percentage) aqueous solution. The fresh oxidant copper hydroxide was

prepared by adding 25 ml of 5% (weight percent) copper sulfate solution to 50 ml of 10% (weight percent) sodium hydroxide solution and mixing immediately. The fresh oxidant copper hydroxide was immediately added to 40 ml of the above 5% (weight percent) alginate oligosaccharide solution, while heated in a boiling water bath until no more brick red precipitates were produced. The reaction system was centrifuged to remove the precipitate to obtain the supernatant. A little supernatant was added to the oxidant again to check whether there was still brick red precipitate produced. If brick red precipitate was still produced, all the supernatants obtained from the centrifugation would continue to react with other part of the oxidant until it was checked that no brick red precipitates were produced. The final reaction system was centrifuged to obtain the supernatant. 4 times the volume of 95% ethanol was added to the supernatant for alcohol precipitation, and the solution was allowed to stand overnight. The solid substance given by alcohol precipitation was filtered and separated, and the solid subtance was washed with absolute ethanol. The obtained solid substance was placed in an oven at 60°C and dried to give the crude alginate oligosaccharide represented by Formula (II).

1g of the crude alginate oligosaccharide was prepared into a 10% (weight percent) aqueous solution, and alcohol precipitation was carried out again by using a 95% ethanol solution. The precipitate obtained by alcohol precipitation again was filtered and separated, followed by optionally washing with absolute ethanol. The precipitate was separated and dried to obtain a solid substance. The solid substance was prepared into a 5% (weight percentage) aqueous solution. The aqueous solution was filtered with a 3 μm pore size membrane and the filtrate was collected. The filtrate was eluted and separated on a molecular exclusion chromatography Bio-Gel-P6 gel column (1.6 x 180 cm, available from Bio-Rad Company). The eluent as mobile phase was 0.2 mol L-1NH_4HCO_3. Eluate from the column chromatography was sequentially collected by using a plurality of 5 ml test tubes, and then the saccharide content of the eluate in each test tube was detected by using a sulfuric acid-carbazole

method. According to the detection results, eluates containing alginate oligosaccharide components with different molecular weights were respectively collected according to the detection results. According to the detection results, eluates containing alginate oligosaccharide components with different molecular weights were respectively concentrated under reduced pressure and lyophilized. Component 1 was discarded, and alginate oligosaccharide components 2-12 were obtained, as shown in Formula (II) (n has a value of 0-10 respectively) with different molecular weights, and alginate oligosaccharide eluent shown in Formula (II) with n=2-8 was collected, combined and dried. Alginate oligosaccharide mixture (tetrasaccharide to decasaccharide mixture) shown in Formula (II) with n=2-8 was produced as a comparative experimental sample.

The proportion of oligosaccharide components with various polymerization degrees in comparative experimental samples was detected by using Superdex peptide (GE Co.) molecular exclusion chromatography combined with multi-angle laser scattering (MALS, Wyatt) . The determination method is the same as the relevant part in example 1. Test results: tetrasaccharide to decasaccharide is represented by dp4-dp10, which is 10% dp4, 12% dp5, 13% dp6, 14% dp7, 15% dp8, 19% dp9 and 17% dp10, respectively.

Products A, B and C respectively prepared in examples 1, 2 and 3, product D in this example and oligosaccharide proportions in the comparative experimental samples are shown in Table 2 below.

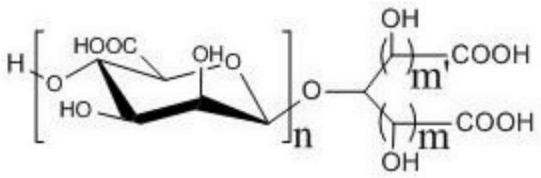
Proportion / Composition	Disaccharide	Trisaccharide	Tetrasaccharide	Pentasaccharide	Hexasaccharide	Heptasaccharide	Octasaccharide	Nonasaccharide	Decasaccharide
A	19%	25%	22%	13%	9%	6%	3%	2%	1%
B	20%	25%	19%	12%	9%	5%	5%	3%	2%
C	8%	20%	28%	19%	13%	6%	3%	2%	1%
D	30%	30%	15%	15%	4%	2%	2%	1%	1%
Comparative samples	0	0	10%	12%	13%	14%	15%	19%	17%

10 g of each of the above four samples A, B, C and D was taken out. The pharmacological activities of these compositions, hexose (6T) and comparative experimental samples were compared according to the method described in "animal model for anti-PD pharmacodynamic evaluation".

The experimental results showed that the latency and climbing-down time of the model group were significantly longer than that of the blank control group. When compared with the model group, the latency and climbing-down time of each dosing group were shortened to different degrees. Among them, the pharmacodynamic activity of products A, B and C are all better than the comparative experimental samples, and are better than the previously expected most active hexasaccharide with single polymerization degree. However, the activity of product D is weaker than hexasaccharide. Without being bound by any theory, it is believed that the proportion of oligosaccharides in the composition has a significant effect on the activity of the product, and adding a certain proportion of disaccharide and trisaccharide has synergistic effect. However, when the proportion of disaccharide and trisaccharide is too high, the activity of the composition would be reduced. See Figures 4-9.

CLAIMS

1. Use of a mannuronic diacid oligosaccharide composition in the manufacture of a medicament for the treatment of Parkinson's disease; wherein the mannuronic diacid oligosaccharide composition comprises mannuronic diacids having Formula (III) or a pharmaceutically acceptable salt thereof:



Formula (III),

wherein n is an integer selected from 1 to 9, m is selected from 0, 1 or 2, m' is selected from 0 or 1, and

wherein,

the total weight of mannuronic diacids wherein n=1-5 accounts for no less than 60% of the total weight of the composition;

the total weight of mannuronic diacids wherein n=1-2 accounts for less than 60% of the total weight of the composition.

2. The use of claim 1, wherein in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacids wherein n=1-2 accounts for 10-50%, preferably 25-50%, of the total weight of the composition.

3. The use of claim 1, wherein in the mannuronic diacid oligosaccharide composition, the ratio of the total weight of mannuronic diacids wherein n=1-3 to the total weight of mannuronic diacids wherein n=4-7 is between 1.0 and 3.5.

4. The use of claim 1, wherein in the mannuronic diacid oligosaccharide composition, the total weight of mannuronic diacids with m+m'=1 or 2 is no less than 50% of the total weight of the composition, preferably 60%-90%, more preferably 70%-90%.

5. The use of claim 4, wherein the total weight of mannuronic diacids with m+m'=1 is no less than 10% of the total weight of the composition, preferably 30-40%.

6. The use of claim 4, wherein the total weight of mannuronic diacids with m+m'=2 is no less than 10% of the total weight of the composition, preferably 30-50%.

7. The use of claim 1, wherein the total weight of mannuronic diacids wherein n=1-5 accounts for 80-95% of the total weight of the composition.

8. The use of claim 1, wherein the total weight of mannuronic diacids wherein n=1-3 accounts for 20-70% of the total weight of the composition.

9. The use of claim 3, wherein the ratio of the total weight of mannuronic diacids wherein n=1-3 to the total weight of mannuronic diacids wherein n=4-7 is between 1.0 and 3.0.

10. The use of any one of claims 1 to 8, wherein the weight percentage content of mannuronic diacids with each of polymerization degrees in the composition is: disaccharide 5-25%, trisaccharide 15-30%, tetrasaccharide 15-28%, pentasaccharide 5-25%, hexasaccharide 2-20%, heptsaccharide 2-20%, octasaccharide 2-20%, nonasaccharide 2-20%, decasaccharide 2-20%.

11. The use of claim 10, wherein the weight percentage content of mannuronic diacids with each of polymerization degrees in the composition is: disaccharide 5-25%, trisaccharide 15-30%, tetrasaccharide 15-28%, pentasaccharide 10-20%, hexasaccharide 5-15%, heptsaccharide 3-10%, octasaccharide 2-5%, nonasaccharide 1-5%, and decasaccharide 1-5%.

12. The use of claim 11, wherein the weight percentage content of mannuronic diacids with each of polymerization degrees in the composition is: disaccharide 10-20%, trisaccharide 18-30%, tetrasaccharide 15-28%, pentasaccharide 10-20%, hexasaccharide 5-10%, heptsaccharide 3-5%, octasaccharide 2-5%, nonaccharide 1-3%, decasaccharide 1-3%.

13. The use of any one of claims 1-11, wherein the pharmaceutically acceptable salt is sodium salt or potassium salt.

14. A method of treating a patient suffering from Parkinson's disease, comprising administering an effective amount of the mannuronic diacid oligosaccharide composition of claim 1-13 to a patient in need thereof.

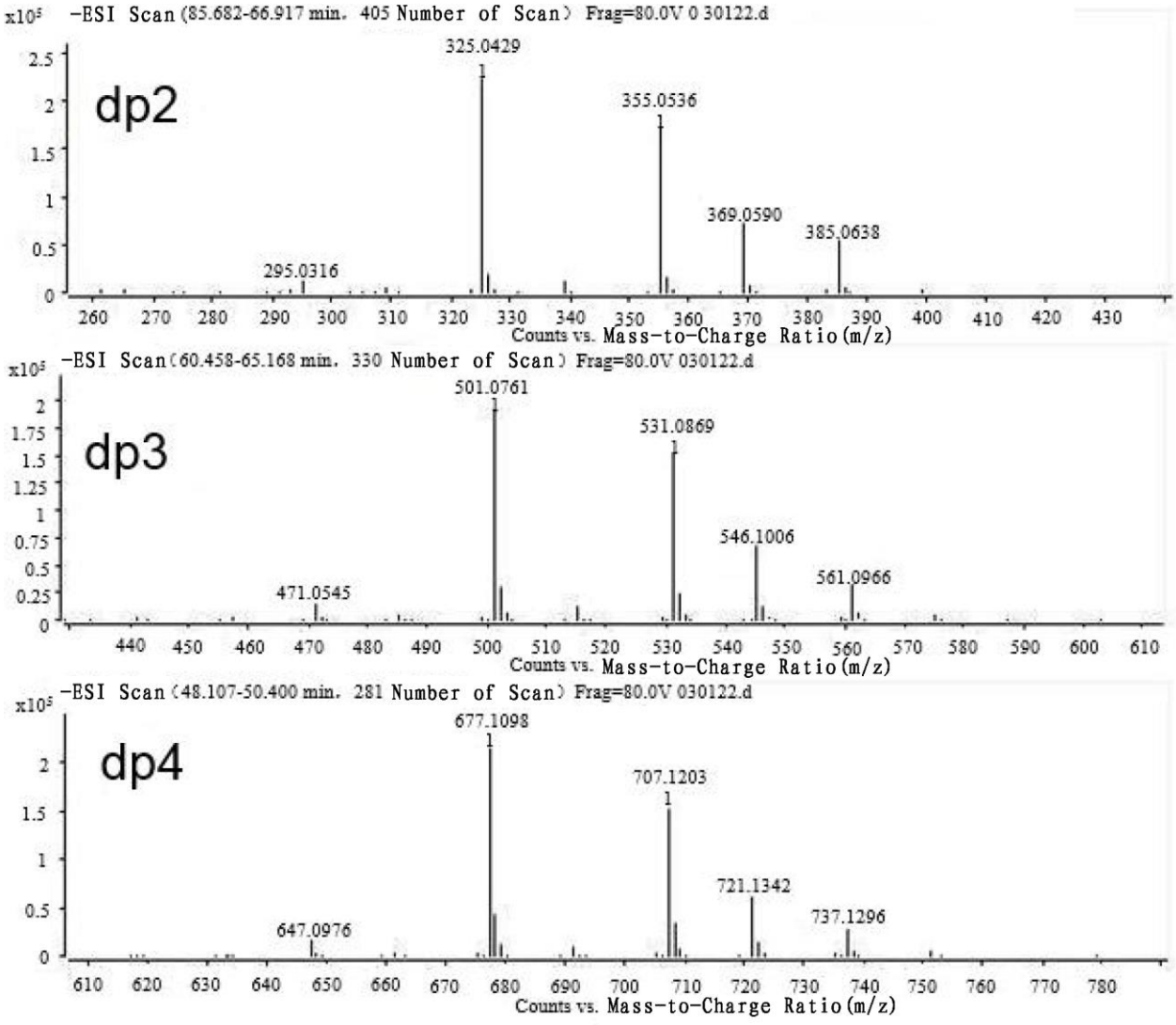

x10⁵ -ESI Scan (85.682-66.917 min, 405 Number of Scan) Frag=80.0V 0 30122.d
dp2
325.0429
355.0536
369.0590
385.0638
295.0316
Counts vs. Mass-to-Charge Ratio (m/z)
x10⁵ -ESI Scan (60.458-65.168 min, 330 Number of Scan) Frag=80.0V 030122.d
dp3
501.0761
531.0869
546.1006
561.0966
471.0545
Counts vs. Mass-to-Charge Ratio (m/z)
x10⁵ -ESI Scan (48.107-50.400 min, 281 Number of Scan) Frag=80.0V 030122.d
dp4
677.1098
707.1203
721.1342
737.1296
647.0976
Counts vs. Mass-to-Charge Ratio (m/z)

Figure 1

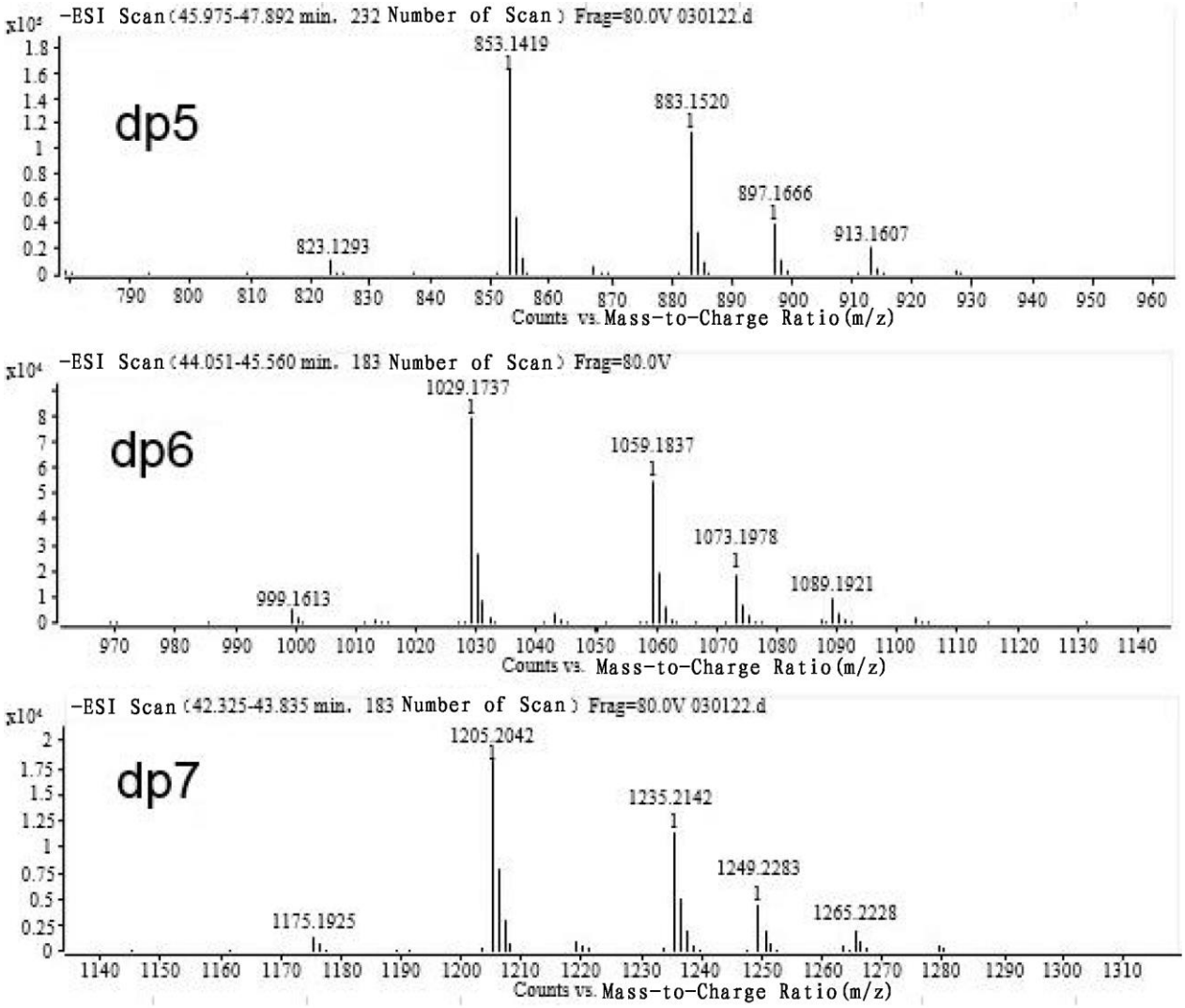

-ESI Scan (45.975-47.892 min, 232 Number of Scan) Frag=80.0V 030122.d
dp5
853.1419
883.1520
897.1666
913.1607
823.1293
Counts vs. Mass-to-Charge Ratio (m/z)
-ESI Scan (44.051-45.560 min, 183 Number of Scan) Frag=80.0V
dp6
1029.1737
1059.1837
1073.1978
1089.1921
999.1613
Counts vs. Mass-to-Charge Ratio (m/z)
-ESI Scan (42.325-43.835 min, 183 Number of Scan) Frag=80.0V 030122.d
dp7
1205.2042
1235.2142
1249.2283
1265.2228
1175.1925
Counts vs. Mass-to-Charge Ratio (m/z)

Figure 2

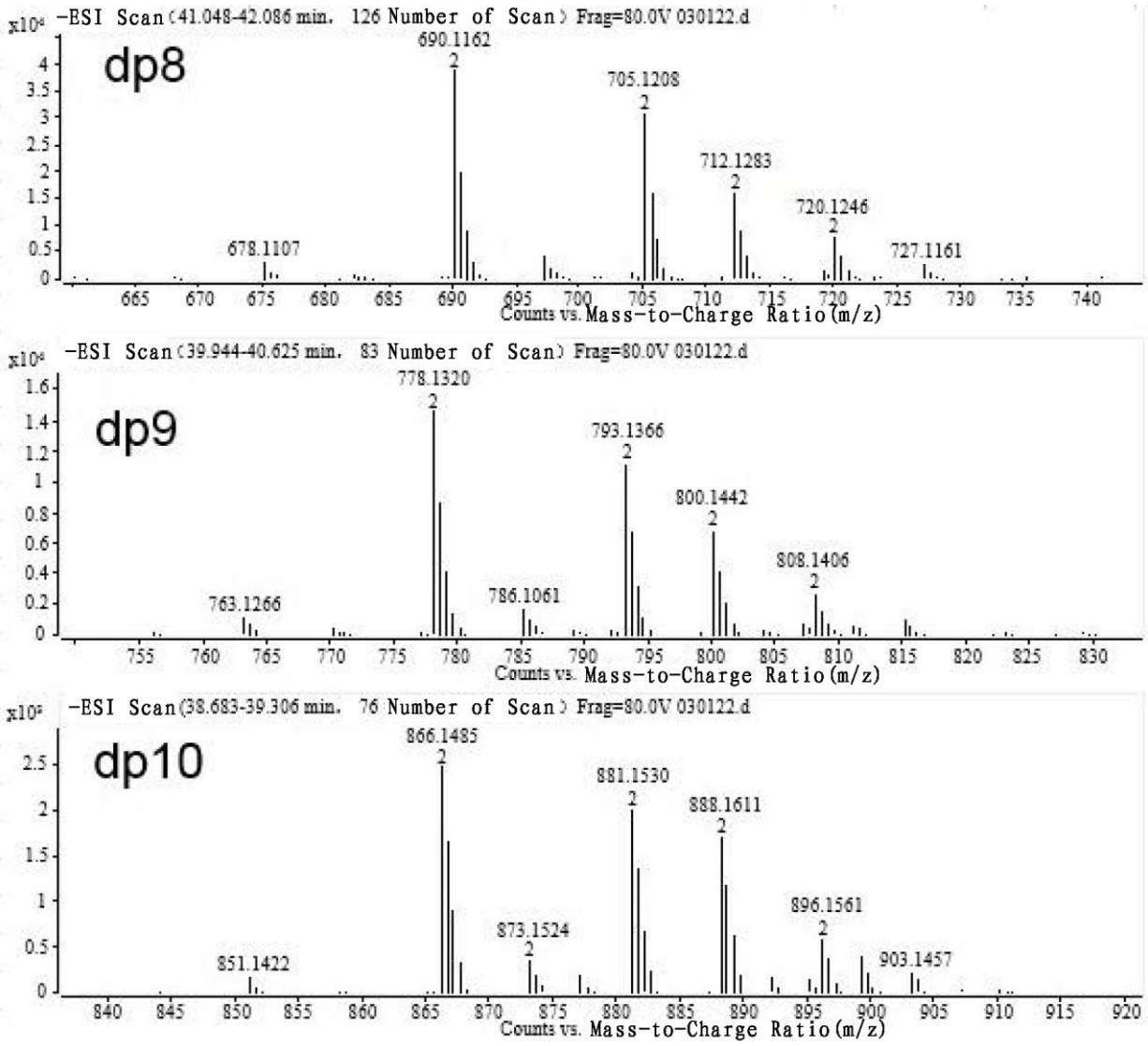

x10⁴ -ESI Scan (41.048-42.086 min, 126 Number of Scan) Frag=80.0V 030122.d
dp8
690.1162
705.1208
712.1283
720.1246
678.1107
727.1161
Counts vs. Mass-to-Charge Ratio (m/z)
x10⁴ -ESI Scan (39.944-40.625 min, 83 Number of Scan) Frag=80.0V 030122.d
dp9
778.1320
793.1366
800.1442
808.1406
763.1266
786.1061
Counts vs. Mass-to-Charge Ratio (m/z)
x10² -ESI Scan (38.683-39.306 min, 76 Number of Scan) Frag=80.0V 030122.d
dp10
866.1485
881.1530
888.1611
896.1561
873.1524
851.1422
903.1457
Counts vs. Mass-to-Charge Ratio (m/z)

Figure 3

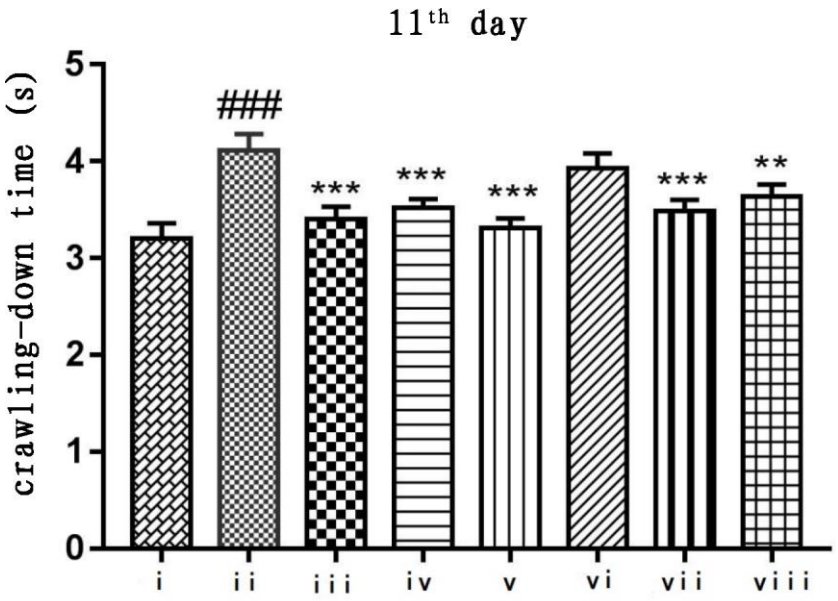


Figure 4

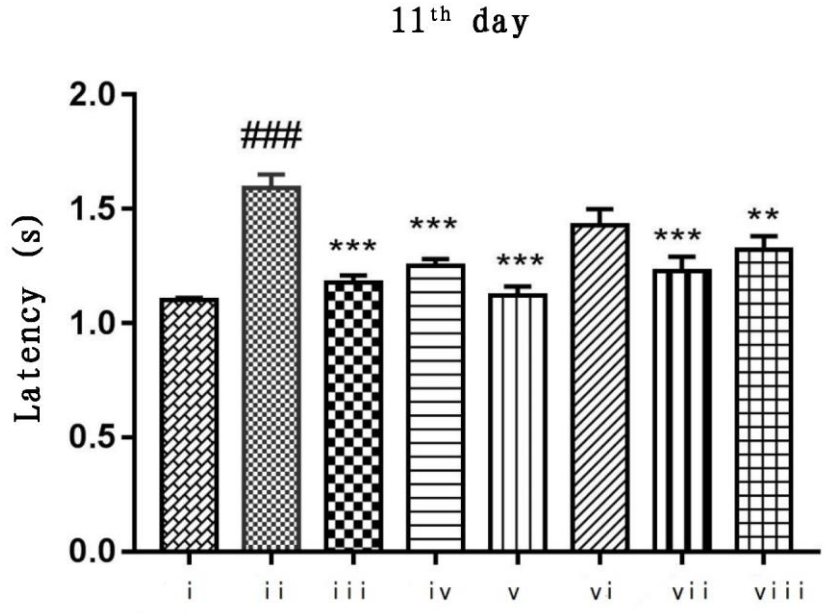


Figure 5

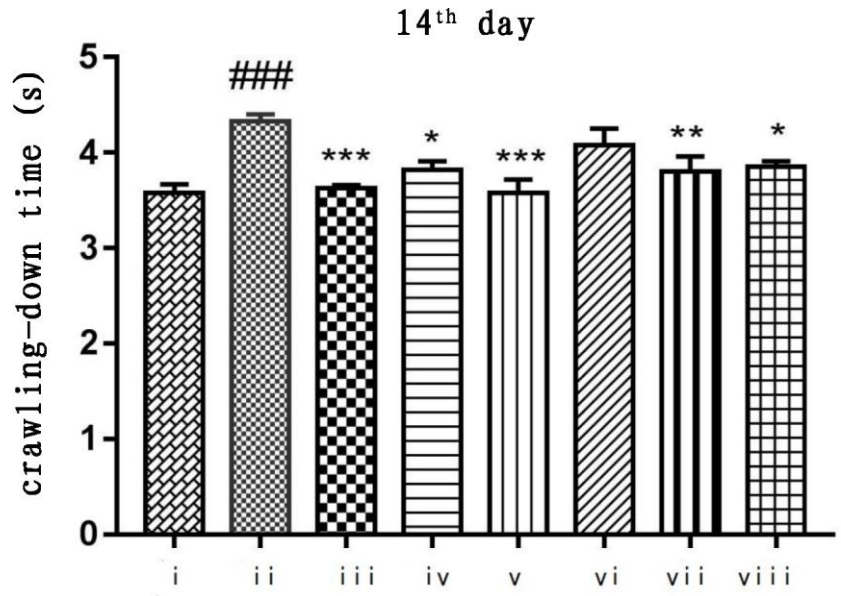


Figure 6

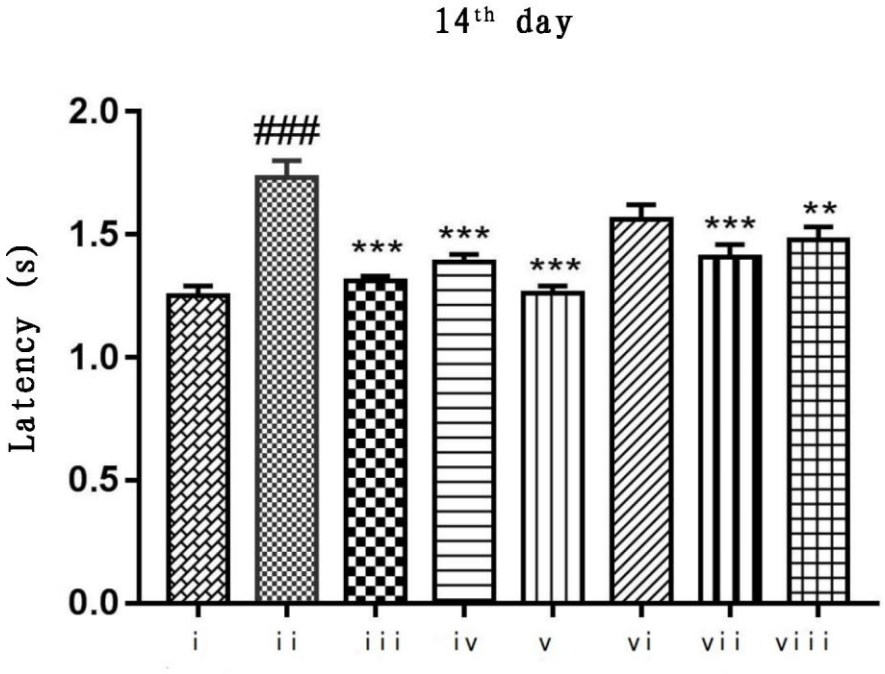


Figure 7

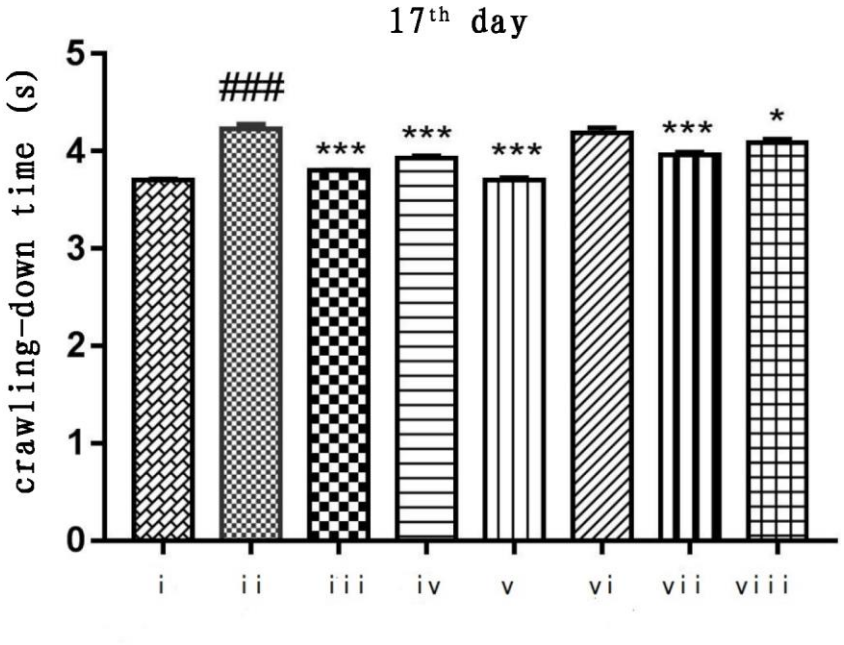


Figure 8

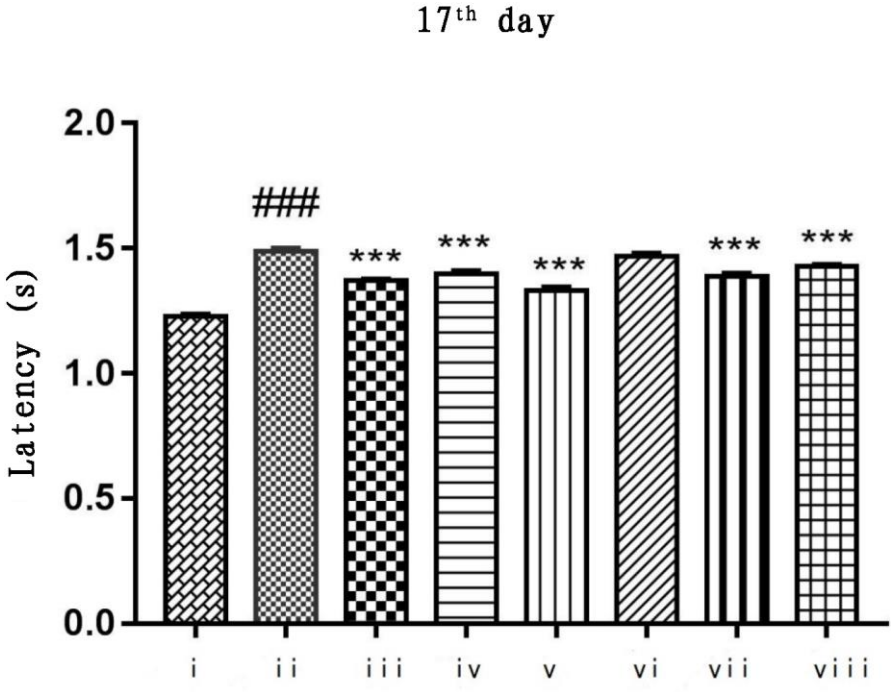


Figure 9