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ORIGINAL ARTICLE

Atractylodis macrocephalae Rhizoma Decoction and its Chemically Profiled Subfractions Alleviate the Side effects of Rhubarb in TCM Pair Medicine

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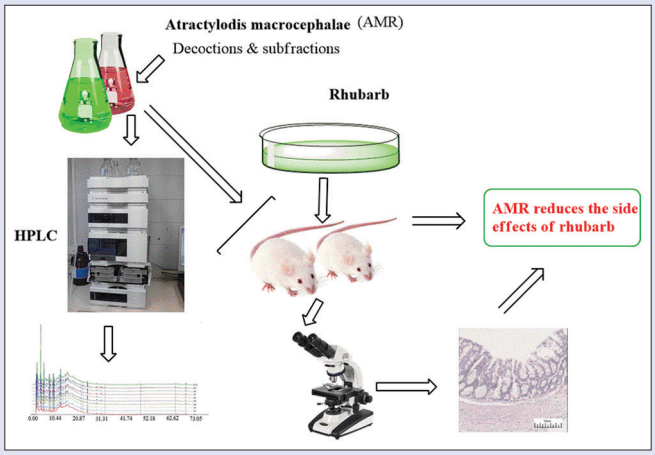
ABSTRACT

Background: Pair medicine is a unique feature of Chinese medicine. It refers to a specific composition of the two drugs usually used in the formulation of composite medicine, which could increase synergistically or decrease the side effects of one medication. *Atractylodis macrocephalae* Rhizoma (AMR), a generally used drug, possessed the spleen-tonifying action always used with Rhubarb as a pair medicine to decrease the side effect of Rhubarb. **Aim:** To investigate the effect of *Atractylodis macrocephalae* Rhizoma (AMR) and its effective constituents to alleviate the Rhubarb's side effect in their pair medicine. **Materials and Methods:** The mice were administered Rhubarb until the induction of diarrhea followed by gastrointestinal injury. The gastrointestinal injured mice were treated with AMR's water decoction and its subfractions for 5 consecutive days. The decoction and subfraction were chemically characterized using the High-performance liquid chromatography (HPLC) with a diode-array detector (DAD) analysis. **Results:** The results showed that AMR's water decoction (6 g/kg) was discovered to be the most effective dose to treat gastrointestinal injury induced by Rhubarb. Body weight, thymus and spleen indexes, the intestinal propulsion rate, and D-xylose absorption in mice with diarrhea and intestinal injury were analyzed to reveal the significant difference between the tested and control groups ($P < 0.01$). The Water Eluated Fraction (WEF), Petroleum Ether Fraction (PEF), and Crude Polysaccharide Fraction (CPF) could not only increase the levels of amylase, gastrin, and vasoactive intestinal peptide significantly but also ease diarrhea and intestinal injury situation compared with the control group ($P < 0.01$). **Conclusion:** AMR and its subfractions effectively ameliorate gastrointestinal side effects of Rhubarb and its components. WEF mainly contained 5-hydroxymethyl furfural and small molecular sugar. PEF mainly composed of sesquiterpene lactone-atractylenolides, while CPF mainly composed of inulin-type oligosaccharides elucidated by HPLC analysis, was the most effective subfraction to alleviate diarrhoea and gastrointestinal injury induced by Rhubarb.

Key words: *Atractylodis macrocephalae* rhizome, diarrhea, fraction, gastrointestinal injury, rhubarb, spleen deficiency

SUMMARY

- The present study aimed to investigate that *Atractylodis macrocephalae* rhizome (AMR) reduces the side effects induced by rhubarb. The decoction and sub-fractions of AMR were prepared. The extracts were chemically characterized using High-performance liquid chromatography with a diode-array detector. Rhubarb was administered to mice until the induction of diarrhea and injury. Then these mice were treated with AMR's water decoction and its subfractions for 5 consecutive days. The histopathological examinations of treated animals confirmed that A. macrocephalae reduces the side effects of Rhubarb in TCM pair medicine



Abbreviations used: AEF: Alcohol Fraction; AMR: *Atractylodes macrocephala* Koidz; AMS: Serum amylase; BN: Batch number; CPF: Polysaccharide Fraction; GAS: Gastrin; IEC-6: Intestinal epithelial crypt 6; IP: Intestinal propulsion; LUTCM: Liaoning University of Traditional Chinese Medicine; PEF: Petroleum Ether Fraction; TCM: Traditional Chinese medicine; VIP: Vasoactive intestinal peptide; VOF: Volatile oil fraction; WEF: Water Fraction.

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INTRODUCTION

Pair medicine is a classic Chinese medicine concept. Two fixed commonly used herbs/medicine are used as a compound formula to synergize the medicinal effect or counteract the side effects compared to a single formula.[1] Rhubarb (*Dahuang* in Chinese), the dried roots and rhizome of *Rheum officinale* Baill,[2] is a frequently employed TCM and was documented in the oldest medicinal plants' treatise

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of < *Shen Nong Ben Cao Jing*.[3] It has been reported as purgative medicine with anthraquinonoids as active constituents. It is reported that long time or signal use can cause various side effects, including injury of the digestive tract and poor absorptive function, and even led to melanosis coli; in higher doses might cause nausea and vomiting.[4] It has also been reported to lower immune function.[5] Thus, Rhubarb is always used with drugs that possessed "tonifying spleen and eliminating dampness" clinically, according to the Chinese medicine theory.[6]

The rhizome of *Atractylodes* m*acrocephala* Koidz. (AMR) is a commonly used Chinese drug and holds various medicinal actions such as revitalising *qi,* tonifying spleen, and treatment of preterm labour.[7] Thus, AMR and Rhubarb are generally regarded as a pair of medicine. The commonly used pair medicine formulations are "*Fang Feng Tong Sheng San*" from < *Yi Xue Qi Yuan*>, "*Zi Shi Dao Zhi Wan*" from < *Nei Wai Shang Bian Huo Lun* > and "*Da Huang Wan*" from < *Sheng Hui* >. In previous studies, the functional modulation of the digestive tract and other pharmacological actions of AMR were explored[8,9] alone. In the present work, the Chinese Medicine pair, AMR-rhubarb compatibility, and the side effect reducing active components were investigated to develop new medicine by fraction compatibility and to know the active component that caused the reduction of side effects.

MATERIALS AND METHODS

Instrument and reagents

The instruments used were Freeze-dryer (Labconco Corporation England), Centrifuge (Thermo Electron Corporation Germany), Microplate Reader (Tecan Group Ltd. Austria), Sonicator (Model KQ-250DE Ultrasonic Instrument Co. Kunshan, China), The Acculab Analytical balance (Sartorius Group Germany), High-speed homogenate (model XHF-F Zhisheng Scientific and Technological Co. Ningbo, China), and Constant temperature incubator (model HPP-9272 Zhisheng Scientific and Technological Co. Ningbo, China).

AMR was collected at Yuqian, Zhejiang, China (Batch No [BN]. 20121025), and Rhubarb was obtained from Anhui Yuhetang Pharmaceutical Co. Ltd, China (BN. 20120703). Both of them were identified as the roots and rhizomes of AMR Koidz and *R. officinale* Baill. by Prof. Wang, Liaoning University of Traditional Chinese Medicine (LUTCM), and their voucher specimens were submitted at Department of Chinese Medicine, LUTCM; D-xylose from Tianjin Bodi Chemical Industrial Co. Ltd. China (BN. 20090916); Saline was bought from Heilongjiang Kelun Pharmaceutical Co. Ltd. China (BN.12060201-2); Activated Carbon from Shenyang Xinxing Chemical Reagent Factory China (BN. 20110619); Methanol (high performance liquid chromatography [HPLC] grade) were purchased from Tiedi Chemical Co., Ltd. USA). Elisa assay kit of VIP and Gastrin (GAS) from RandD Systems (BN.201306, USA); Assay kit of D-xylose and AMS from Nanjing Jiancheng Bioengineering Institute China (BN. 20130325, 20131203.

Preparation of herbal extracts

Preparation of rhubarb decoction

500 g of Rhubarb was pulverized and immersed in the water thoroughly, then boiled 10 min once for three times, and then, all fractions were evaporated in vacuum to 1 g/ml and stored at 4°C.

Preparation of *Atractylodes macrocephala* decoction

1000 g of AMR was powdered and boiled with water 1 h twice, and after filtration, the extracts were concentrated in vacuum to 0.5 g/mL and stored at 4°C.

Table 1: The yield and the dose of the sub-fraction of *Atractylodes macrocephala* Koidz

Group	Sub-fraction	Yield (%)	Dose (g/kg)
Group 4	WEF	32.70	1.964
Group 5	AEF	0.8580	0.05128
Group 6	VOF	0.8640	0.05184
Group 7	PEF	0.2045	0.01233
Group 8	CPF	36.66	2.200

WEF: Water fraction, AEF: Alcohol fraction, VOF: Volatile oil fraction, PEF: Petroleum ether fraction, CPF: Crude polysaccharide fraction

Preparation of the subfractions of *Atractylodes macrocephala*

The separation of AMR decoction was performed following its polarity difference of components. Multi-mode fractionation methods were used to prepare AMR fractions. The yield of every fraction is shown in Table 1. Meanwhile, the volatile oil fraction (VOF), crude polysaccharide fraction (CPF), petroleum ether fraction (PEF), water fraction (WEF), and alcohol fraction (AEF) were further separated per our previous procedure slightly modified[10] given in Figure 1.

Subfraction characterization

The subfractions were characterized chemically with the use of acetonitrile and water system using an Agilent 1260 with HPLC with A Diode Array Detector and a Kromasil C_{18} column was used. The gradient elution of acetonitrile and water from 3% acetonitrile to 100% at 242 nm as maximum absorptive wavelength.

Animals

Male mice with good health (20.0 ± 2 g) were acquired from Changsheng Bio-Technique Co. Limited, identification number SCXK 2013-0009. Mice were housed at laboratory-controlled temperature and humidity, free access to food and water; all experimental protocols were strictly followed according to the guidelines for the use and care of laboratory animals,[11,12] and complied with the current ethical regulations for animal research of LUTCM.

Preparation of the reference reagents

Five percent carbon powder suspension: 7 g Arabic gum powder was added with 60 mL purified water and boiled until the solution's transparency. Then 3.5 g activated carbon powder was added to the above solution and boiled.

Five percent D-xylose solution: 2 g D-xylose was dissolved with 40 mL purified water to make the 5% D-xylose solution.

Administration of rhubarb and *Atractylodes macrocephala*

Mice were orally administered with 20 g/kg of 100% rhubarb decoction for 8 days. During the time, the mice showed weight loss, sluggish, loosed stools, and tarnished fur.

Animal groups

Test 1: The 60 rhubarb fed mice were divided into six groups (n = 10 per group) randomly, and each group was administered, *i.g.*, according to the following manner: Group 1: (control), Group 2: 0.2 mL/10 g body weight of water, Group 3–5: 2 g/kg, 10 g/kg,

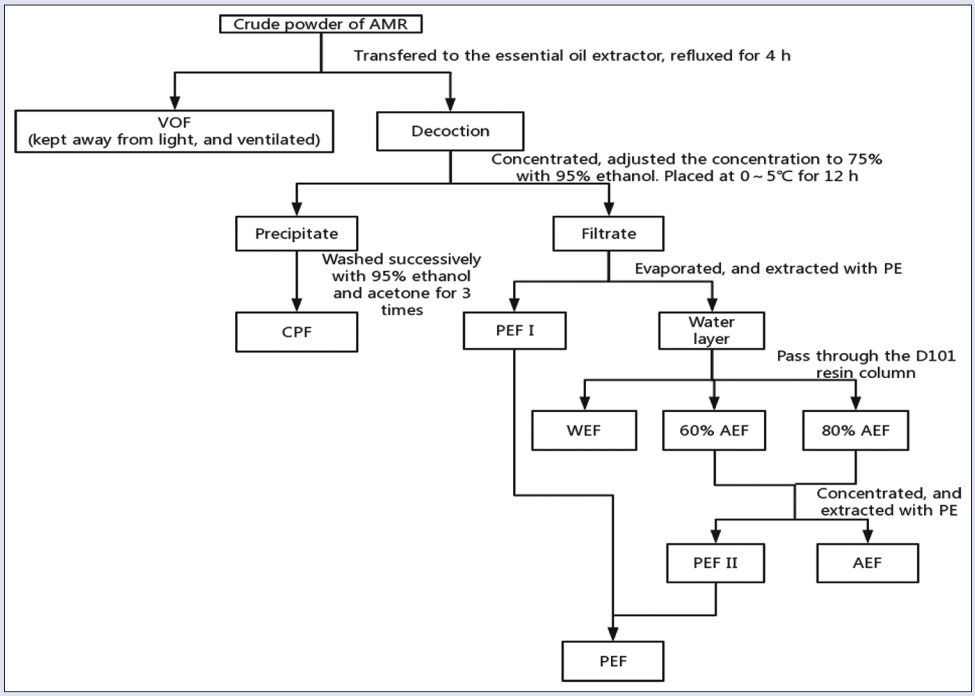

Figure 1: Scheme of *Atractylodes macrocephala* fractionation (Crude Polysaccharide Fraction), Petroleum Ether Fraction, Water Eluated Fraction, Alcohol Eluated Fraction

20 g/kg body weight of AMR decoction, respectively. Group 6 was fed standard drug Smecta with a daily dose of 1.5 g/kg. On the 5th day of drug administration, organ indexes were measured, and each mouse was tested for designated properties.

Test 2: The Rhubarb fed mice were randomly divided into four groups (n = 10 per group). These groups were treated for 5 days as Group 1 (control), 2 (model): 0.2 mL/10 g body weight of water; Group 3–4: 6 g/kg, 10 g/kg body weight of AMR decoction, respectively. Each mouse was tested for some designated items.

Test 3: The Rhubarb fed mice were divided into nine groups randomly (n = 10 per group). Each group was treated for 5 days as Group 1 (control), 2 (model): 0.2 mL/10 g body weight of 1% tween-80 steamed water solution; Group 3: 6 g/kg body weight of AMR decoction; Group 4, 5, 6, 7, and 8: the dose of the subfractions is given in Table 1 according to 6 g/kg crude drug; Group 9: 1.5 g/kg body weight of Smecta montmorillonite powder. Each mouse was tested for some designated items. All subfractions were dissolved in a 1% between 80 steamed water solution.

Test 4: The Rhubarb fed mice were divided into nine groups randomly (n = 10 per group). Each group was treated for 5 days as Group 1 (control); Group 2 (model): 0.2 mL/10 g body weight of 1% tween-80 steamed water solution; Group 3 and 4: 1.964, 0.9820 g/kg body weight of WEF respectively; Group 5 and 6: 0.01233, 0.006165 g/kg body weight of PEF respectively; Group 7 and 8: 2.200, 1.100 g/kg body weight of CPF, respectively; 1% between 80 steamed water solution was used as vehicle for fractions. Group 9: 1.5 g/kg body weight of Smecta montmorillonite powder. Each mouse was tested for designated items.

Measurement of the biochemical indexes

Biochemical indexes were measured according to the previous publications;[13,14] after fasting for 24 h, mice in each group were administered 5% D-xylose solution (0.2 mL/10 g body weight). Blood was collected from retroocular venous plexus after 1 h of the drug administration. Centrifugation at 3500 rpm for 10 min was used to separate the serum. The content of D-xylose, AMS and GAS were determined in the serum by ELISA method. Then each group was administered 5% carbon powder suspension with 0.2 mL/10 g body weight. 30 min later, mice were operated to execute to ligate the lower end of pylorus and the ileocecus. Then, the small intestines were taken and put on the plank. The length from the lower end of the pylorus to the carbon powder's front edge and the whole small intestines' length was measured. The spleens and thymus's entire tissues were weighted after removing their tissue fluid and blood by filter papers. The GAS, AMS in serum, and VIP in the colon were measured in Test 4. After fasting for 24 h with *ad libitum* of water, then the mice were sacrificed. The liver, spleen, stomach, and colon tissue was taken and then washed with PBS. The tissue slices were observed under an electron microscope and photographed.[15]

Statistical analysis

Statistical Product and Service Solution 20.0 was used in the statistical evaluation by the analysis of variance. Differences with an associated $P < 0.05$ were considered statistically significant.

The dose selection of *Atractylodes macrocephala* water decoction

As shown in Supplementary Materials, Figures S1 and S2, weight loss, sluggish, loose stools, and skin tarnished were measured and observed for all the mice. Subsequently, all AMR and subfractions fed mice data were compared with standard model symptoms. Results indicated that the Rhubarb could effectively cause diarrhea and intestinal injury in an animal.

The dose selection of AMR water decoction was performed in two tests. Test 1: Effects of different doses of AMR water decoction on gastrointestinal function and immune organs in mice with spleen deficiency and diarrhea; Test 2: Effects of the lowest effective dose of AMR water decoction gastrointestinal function and immune organs in mice with spleen deficiency and diarrhea. In Test 1, animals were administered, *i.g.*, with 2 g/kg, 10 g/kg, and 20 g/kg body weight of AMR water decoction, respectively; moreover, in Test 2, 6 g/kg, and 10 g/kg body weight of AMR water decoction were given, respectively. Test 1 showed that the AMR water decoction (10 g/kg) showed significant effects on intestinal propulsion and serum D-xylose content. Test 2 showed that the AMR water decoction (6 g/kg) showed a more significant effect on intestinal propulsion and serum D-xylose content than that of the AMR water decoction (10 g/kg). Therefore, the 6 g/kg of AMR water decoction was determined to be the most effective dose to treat gastrointestinal injury induced by Rhubarb. The dose selection of AMR water decoction (the most effective dose) to treat the gastrointestinal injury caused by Rhubarb was described in the Supplementary Materials Figures S1-S10. Thus, 6 g/kg of AMR water decoction was selected as the dose to further examine its action for the side effects of Rhubarb, and the doses of subfractions of AMR water decoction were calculated as equivalent to the 6 g/kg of AMR water decoction based on their separation recoveries.

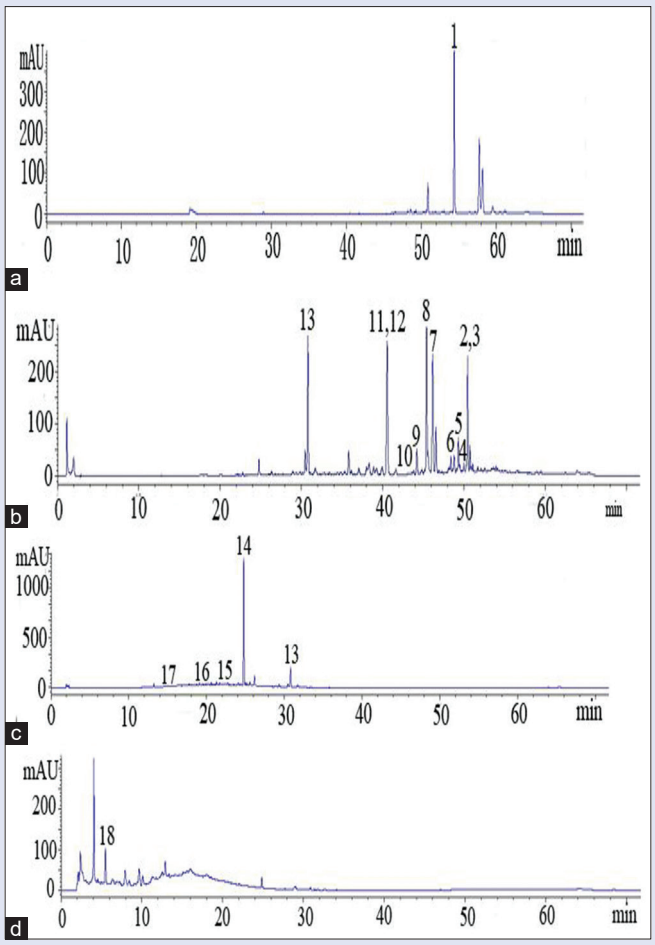

Figure 2: High performance liquid chromatography chromatogram of *Atractylodes macrocephala* subfractions (a) Volatile oil fraction; (b) Petroleum Ether Fraction; (c) Alcohol Fraction; (d) Water Fraction; Compounds: 1. Atractylone; 2. Dibutyl phthalate; 3. Diisobutyl phthalate; 4. Juniper camphor; 5. Atractylenolide II; 6. Sitosterin; 7. Isoatractylenolide I; 8. Atractylenolide I; 9. Stigmasterol; 10. 3 β-acetoxy-atractylenolide I; 11. Atractylenolide III; 12. Taraxeryl acetate; 13.(4E, 6E and 12E)-tetradeca-4, 6, 12-triene-8, 10-diyne-1, 3, 14-triol; 14.(4E, 6E, 12E)-3, 14-dihydroxytetradeca-4, 6, 12-trien-8, 10-diyn-1-yl acetate; 15. 5-Hydroxymethyl furfural ether; 16. Caprolactam; 17. Atractyloside A; 18. 5-Hydroxymethyl furfural

RESULTS

Subfraction characterization and high-performance liquid chromatography finger prints

The main components of VOF after HPLC-DAD analysis were monoterpenes and sesquiterpenes; the PEF showed sesquiterpene lactone as major component; the ethanol fraction confirmed polyacetylene as major secondary metabolite; 5-hydroxymethyl furfural, and small molecular saccharides were identified in the WEF, and the inulin-type oligosaccharides in CPF. The identified compounds peaks of the subfractions are given in Figure 2. Moreover, structures of the compounds in Supplementary file Table S1, whereas the fingerprints of these subfraction are given in Supplementary Materials Figure S11.

Effect on mice body weight of *Atractylodes macrocephala* and its subfractions

As shown in Figure 3, mice's body weight of the subfractions treated group was higher significantly than the model group. As shown in Figure 4, before medication, mice's body weight in each group was markedly varied from that in the control group ($P < 0.01$). The effective subfractions of AMR were administered *i.g.*, for 5 days, the body weight of mice in the model group was significantly different from the control group ($P < 0.01$). There was still a difference between the weight of group (C)-group (H) and that of the model group, indicating that the body weight increased to a certain extent after administration of AMR and its subfractions.

Effect of *Atractylodes macrocephala* and subfractions on mice immune organ index

As shown in Figure 5, the thymus index in the model group decreased compared with the control group; there were significant differences among the Mendelian randomization and its subfractions fed groups and the model group ($P < 0.01$). The thymus index in the AMR water decoction, PEF, and CPF groups was significantly higher than the model group ($P < 0.01$), AEF group was higher than the model group ($P < 0.05$). The spleen index of model group decreased compared with the control group ($P < 0.01$). The spleen index of the AMR water decoction and CPF group was significantly higher than the model group ($P < 0.01$), WEF and PEF group were higher than the model group ($P < 0.05$). As shown in Figure 6, the thymus index of the model group decreased compared with the control group ($P < 0.01$). The thymus index in the WEF group (C), PEF group (E), PEF group (F), CPF group (G), and CPF group (H) was higher significantly than the model group ($P < 0.01$). The spleen index of the model group was lower than the control group ($P < 0.01$). The spleen index in the WEF group (C), PEF group (E), CPF group (G), and CPF group (H) was significantly higher than the model group ($P < 0.01$), the PEF group (F) was higher than model group ($P < 0.05$).

Effect of *Atractylodes macrocephala* and subfractions on mice intestine propulsion

As shown in Figure 7, the model group was lower than that in the control group ($P < 0.05$); the WEF group was higher than that in the model

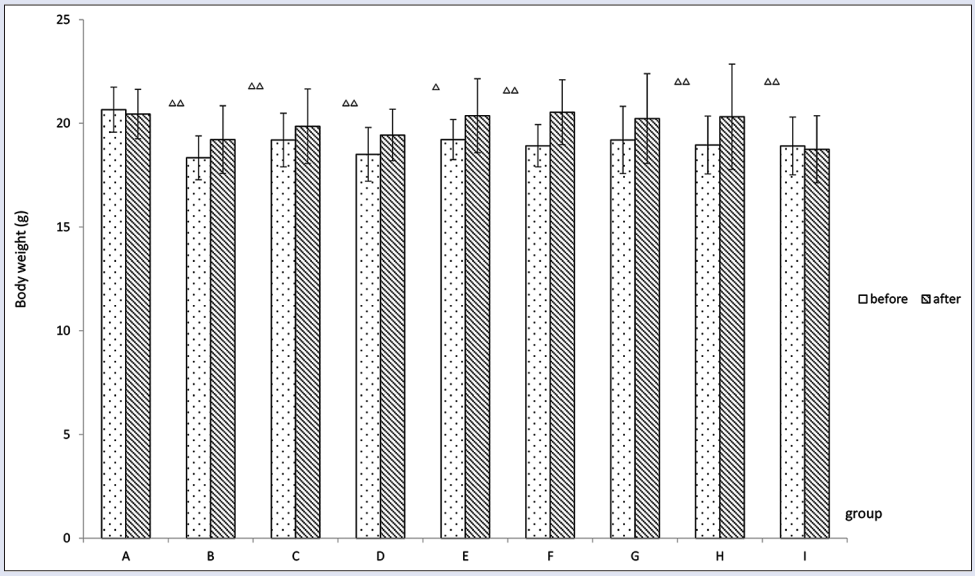

Figure 3: Effect on body weight of *Atractylodes macrocephala* and subfractions. Control group (A); model group (B); water decoction (C); Water Fraction (D); Alcohol Fraction (E); Volatile oil fraction (F); Petroleum Ether Fraction (G); Crude Polysaccharide Fraction (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

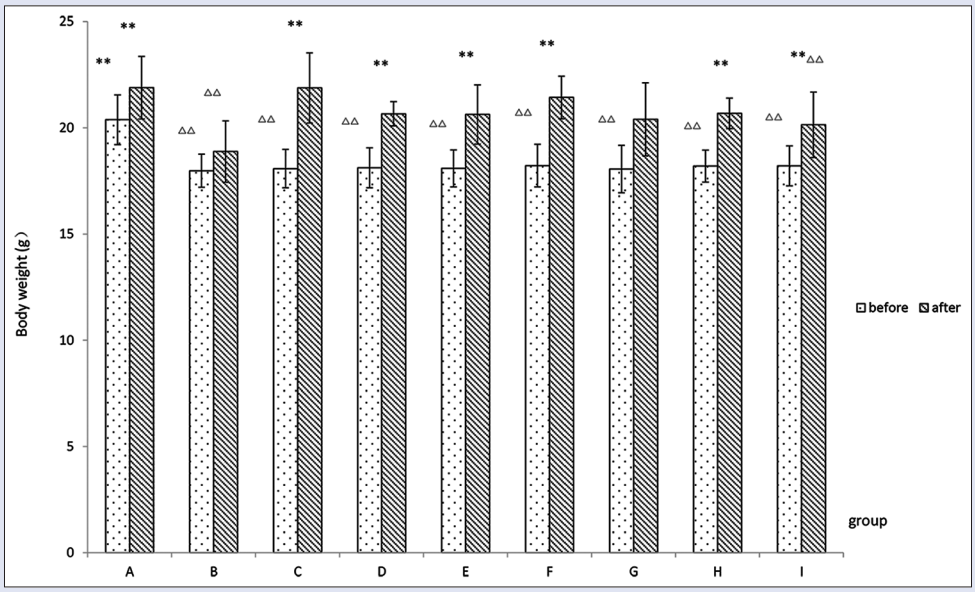

Figure 4: Effect on body weight of the effective subfractions in *Atractylodes macrocephala*. Control group (A); model group (B); Water Fraction (1.964 g/kg) group (C); Water Fraction (0.982 g/kg) group (D); Petroleum Ether Fraction (0.01233 g/kg) group (E); Petroleum Ether Fraction (0.006165 g/kg) group (F); Crude Polysaccharide Fraction (2.200 g/kg) group (G); Crude Polysaccharide Fraction (1.100 g/kg) group (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

group ($P < 0.01$), the AMR water decoction, PEF, and CPF group were all higher than that in model group ($P < 0.05$).

Effect of *Atractylodes macrocephala* and subfractions on serum D-xylose content

As shown in Figure 8, the model group was lower than that in the control group ($P < 0.01$); the AMR water decoction, WEF, PEF, and CPF group were all higher than that in the model group ($P < 0.01$). As shown in Figure 9, the model group was lower than that in the control group ($P < 0.01$); compare with the model group, there was a significant difference in the CPF group (H) ($P < 0.05$) and there were significant differences in WEF group (C), PEF group (E), and CPF group (G) ($P < 0.01$).

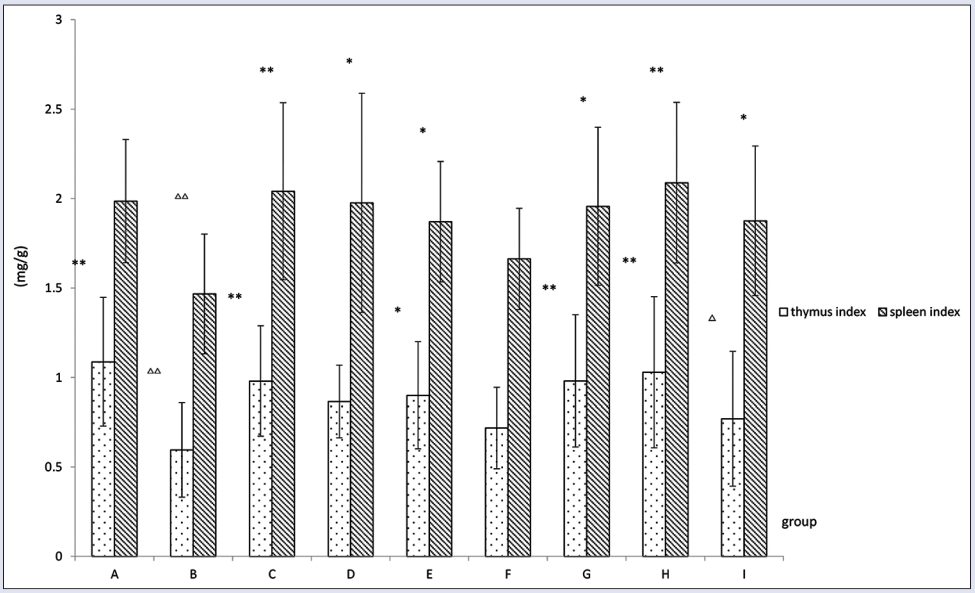

Figure 5: Effect of *Atractylodes macrocephala* and subfractions on mice immune organ index. Control group (A); model group (B); water decoction (C); Water Fraction (D); Alcohol Fraction (E); Volatile oil fraction (F); Petroleum Ether Fraction (G); Crude Polysaccharide Fraction (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

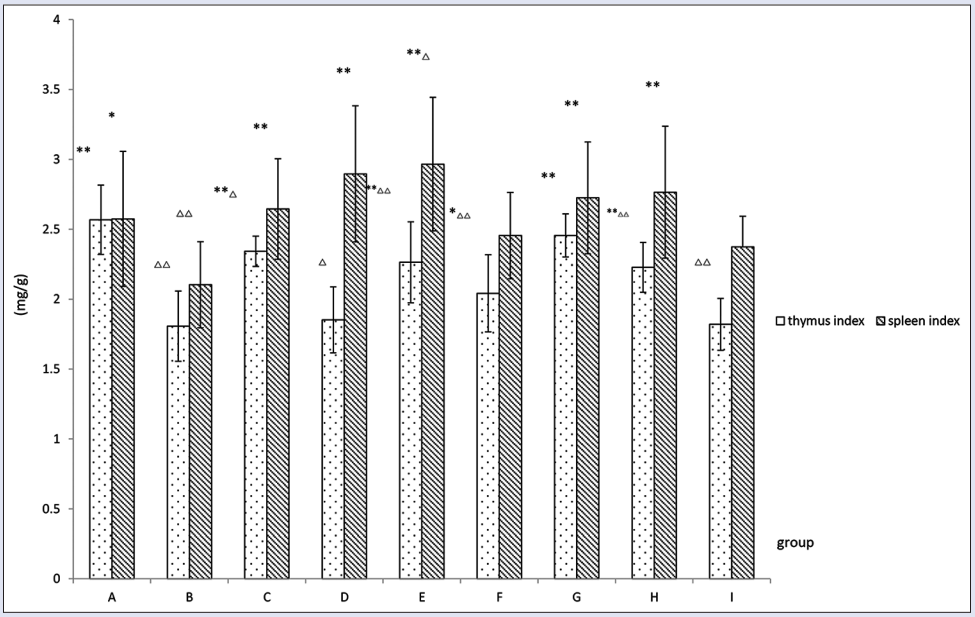

Figure 6: Effect of the effective subfraction of *Atractylodes macrocephala* on mice immune organ index. Control group (A); model group (B); Water Fraction (1.964 g/kg) group (C); Water Fraction (0.982 g/kg) group (D); Petroleum Ether Fraction (0.01233 g/kg) group (E); Petroleum Ether Fraction (0.006165 g/kg) group (F); Crude Polysaccharide Fraction (2.200 g/kg) group (G); Crude Polysaccharide Fraction (1.100 g/kg) group (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

Effect of *Atractylodes macrocephala* subfractions on serum amylase

As shown in Figure 10, compared with the control group, the level of serum amylase (AMS) was significantly lower ($P < 0.01$) in model group (B), WEF group (C), WEF group (D), PEF group (E), PEF group (F), and CPF group (H). In comparison with the model group, WEF group (C), PEF group (E), CPF group (G), and CPF group (H), the AMS level was significant ($P < 0.01$).

Effect of *Atractylodes macrocephala* subfractions on gastrin

As shown in Figure 11, all groups with subfractions and model groups have lower GAS content than the control group ($P < 0.01$) after the model cause

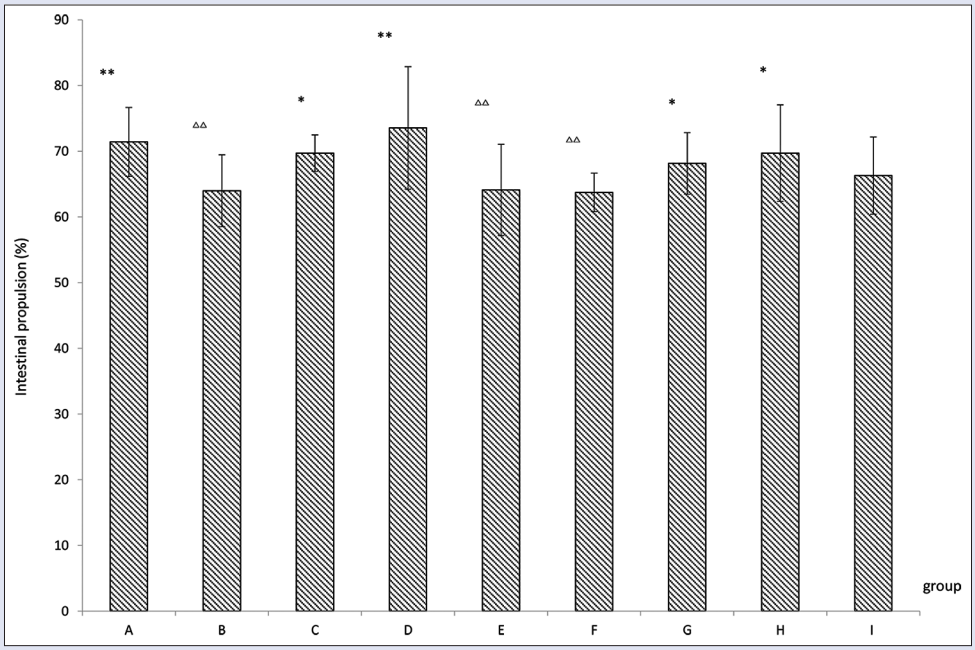

Figure 7: Effect of *Atractylodes macrocephala* and subfractions on mice intestine propulsion. Control group (A); model group (B); water decoction (C); Water Fraction (D); Alcohol Fraction (E); Volatile oil fraction (F); Petroleum Ether Fraction (G); Crude Polysaccharide Fraction (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

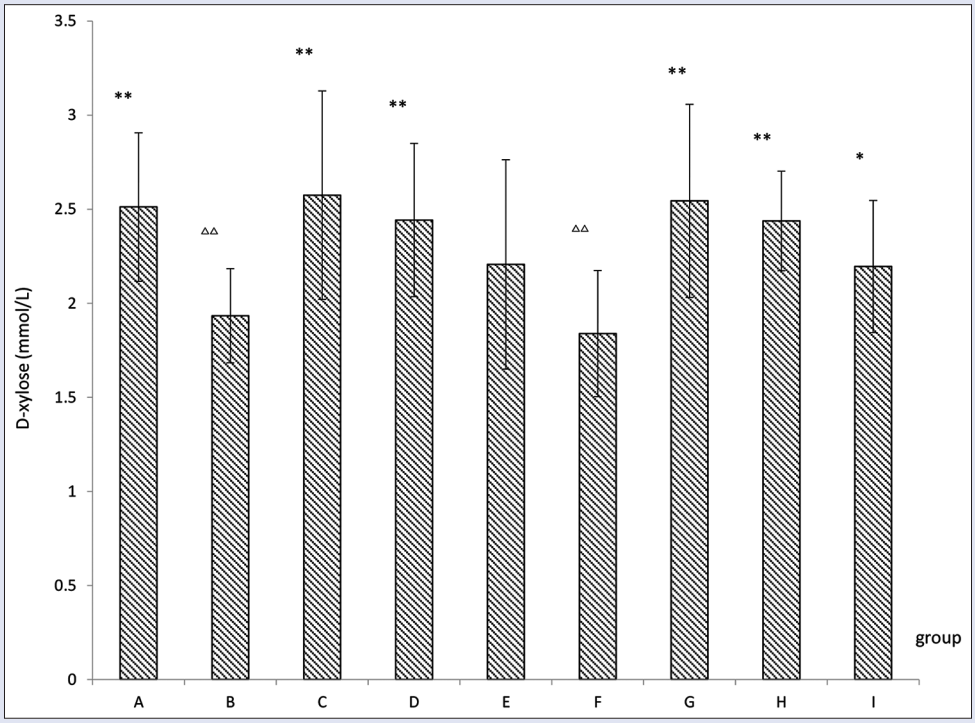

Figure 8: Effect of *Atractylodes macrocephala* and subfractions on serum D-xylose content. Control group (A); model group (B); water decoction (C); Water Fraction (D); Alcohol Fraction (E); Volatile oil fraction (F); Petroleum Ether Fraction (G); Crude Polysaccharide Fraction (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

of spleen deficiency. GAS content dropdown more conspicuously. The WEF group (C), CPF group (G), and CPF group (H) had significantly different as compared with the model group ($P < 0.01$), whereas the effect of WEF group (D), PEF group (E), and PEF group (F) was less obvious ($P > 0.05$).

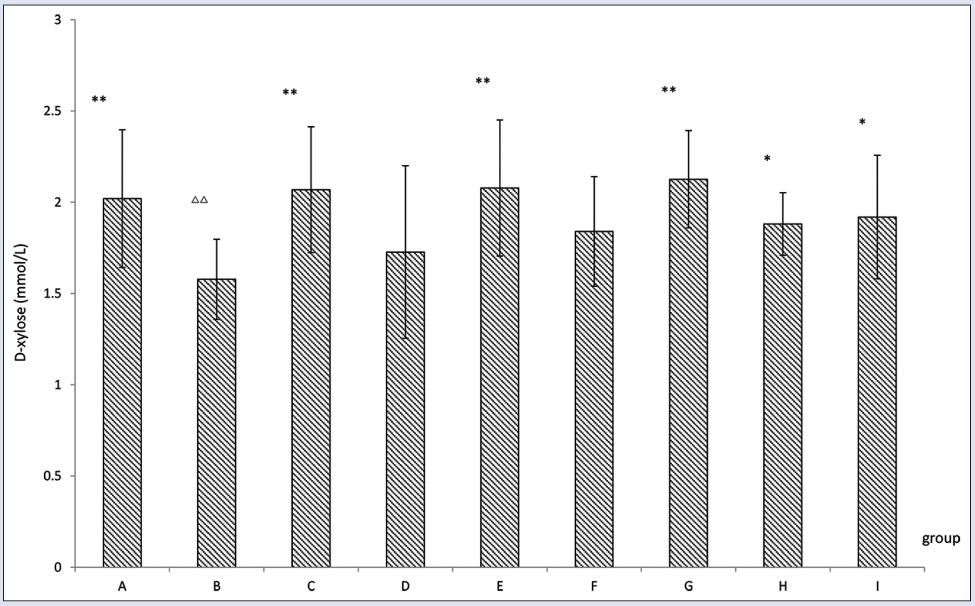

Figure 9: Effect of the effective subfractions of *Atractylodes macrocephala* on serum D-xylose content. Control group (A); model group (B); Water Fraction (1.964 g/kg) group (C); Water Fraction (0.982 g/kg) group (D); Petroleum Ether Fraction (0.01233 g/kg) group (E); Petroleum Ether Fraction (0.006165 g/kg) group (F); Crude Polysaccharide Fraction (2.200 g/kg) group (G); Crude Polysaccharide Fraction (1.100 g/kg) group (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

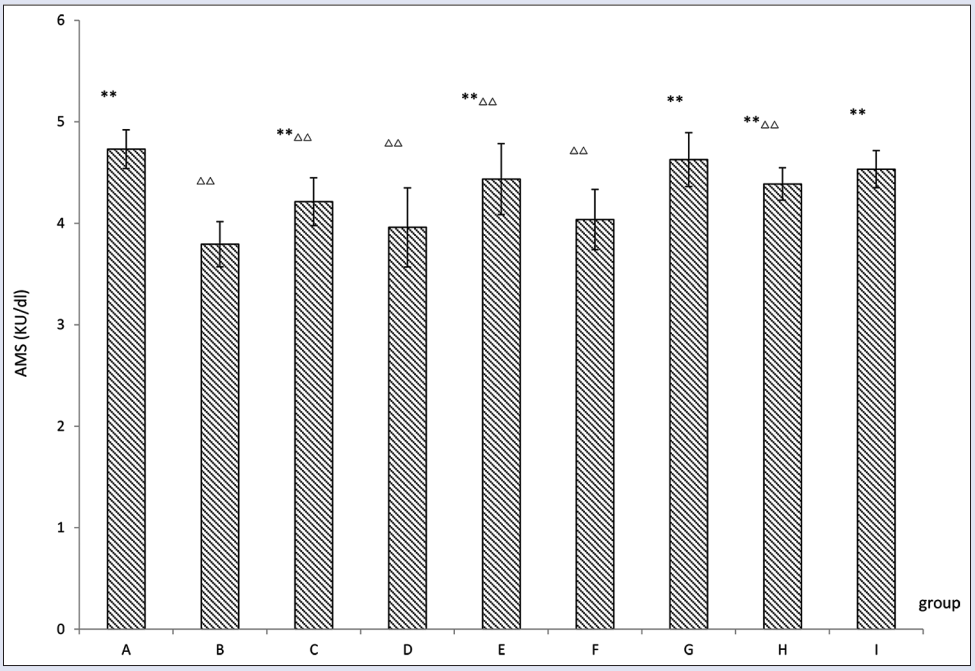

Figure 10: Effect of *Atractylodes macrocephala* subfractions on AMS. Control group (A); model group (B); Water Fraction (1.964 g/kg) group (C); Water Fraction (0.982 g/kg) group (D); Petroleum Ether Fraction (0.01233 g/kg) group (E); Petroleum Ether Fraction (0.006165 g/kg) group (F); Crude Polysaccharide Fraction (2.200 g/kg) group (G); Crude Polysaccharide Fraction (1.100 g/kg) group (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

Effect of *Atractylodes macrocephala* subfractions on vasoactive intestinal peptide

As shown in Figure 12, the model group's vasoactive intestinal peptide (VIP) content was lower than that in the control group ($P < 0.01$). The PEF group (E), PEF group (F), CPF group (G), and CPF group (H) had more significant differences as compared with the model group ($P < 0.01$). While compared with the model group, the WEF group (C) showed an increasing trend ($P < 0.05$).

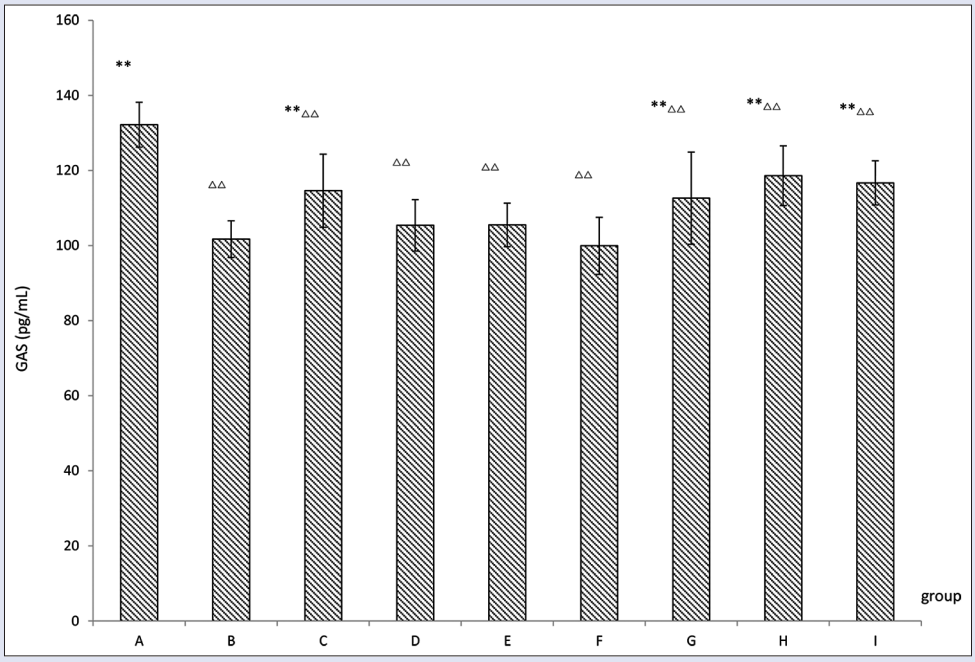

Figure 11: Effect of *Atractylodes macrocephala* subfractions on serum gastrin. Control group (A); model group (B); Water Fraction (1.964 g/kg) group (C); Water Fraction (0.982 g/kg) group (D); Petroleum Ether Fraction (0.01233 g/kg) group (E); Petroleum Ether Fraction (0.006165 g/kg) group (F); Crude Polysaccharide Fraction (2.200 g/kg) group (G); Crude Polysaccharide Fraction (1.100 g/kg) group (H); positive drug group (I); Cumulative values are reported as Mean ± standard deviation for 10 mice in each group, $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

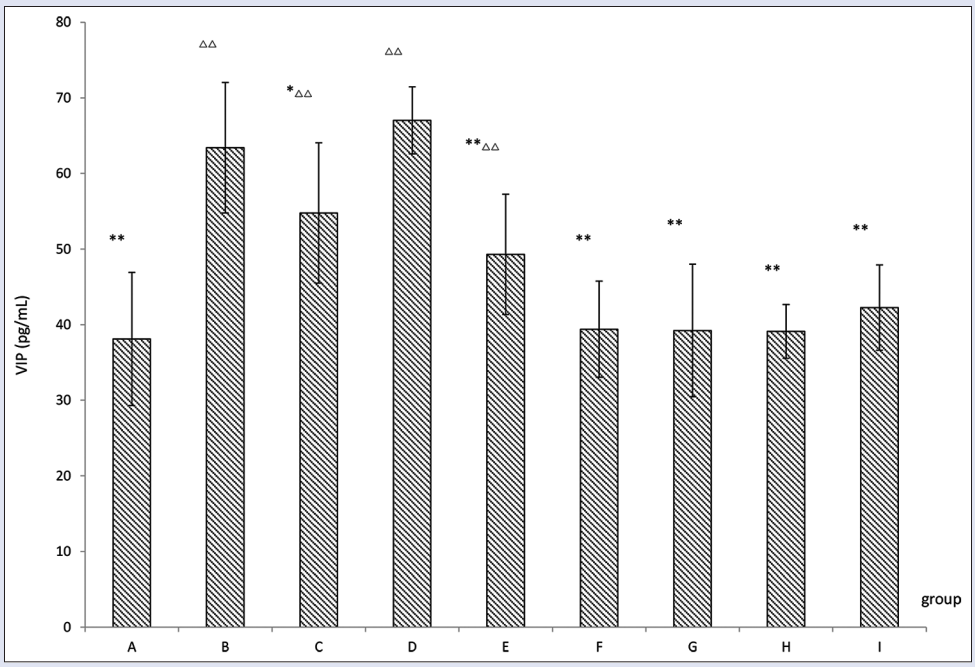

Figure 12: Effect of *Atractylodes macrocephala* subfractions on Vasoactive intestinal peptide. Control group (A); model group (B); Water Fraction (1.964 g/kg) group (C); Water Fraction (0.982 g/kg) group (D); Petroleum Ether Fraction (0.01233 g/kg) group (E); Petroleum Ether Fraction (0.006165 g/kg) group (F); Crude Polysaccharide Fraction (2.200 g/kg) group (G); Crude Polysaccharide Fraction (1.100 g/kg) group (H); positive drug group (i); $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with model group

Histopathological findings in hepatic tissues

The protective effects of the AMR and subfractions can easily be seen; the hepatocellular structure is typical in the control group. In the model group, the structure changes and hyperplasia can be seen; However, AMR protective effect may also be observed in Figure 13 a-i. for details description of each test read section 5.1 Supplementary Materials.

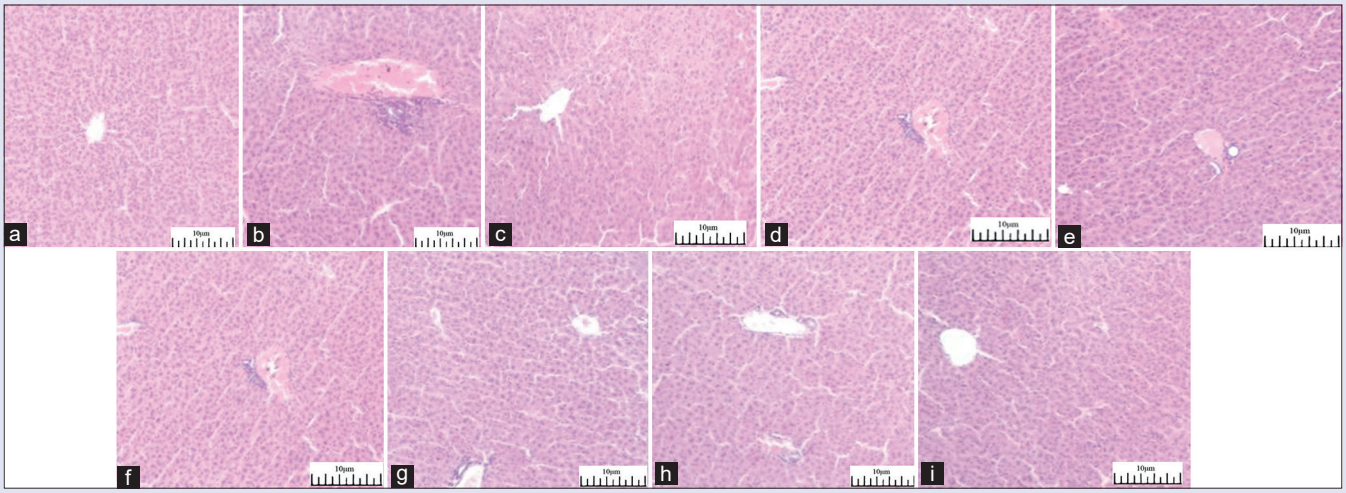

Figure 13: (a-i) Histopathological findings in hepatic tissues

Histopathological findings in spleen tissues

Control group: Mice have no abnormal change in spleen tissue membrane, trabecular, the red pulp cells, and white pulp cells [Figure 14a]. Model group: Compared with control group, mice have thickening of spleen tissue membrane; spleen trabecular with swelling; the red pulp cells and white pulp cells of uneven coloring, which with fuzzy boundaries and aggregation into blocks; around the posterior artery gathered a large number of inflammatory cells infiltration [Figure 14b]. Positive drug group: compared with model group, spleen trabecular, the red pulp cells, and white pulp cells of mice recovery in good condition. However, around the posterior artery still gathered some inflammatory cells infiltration [Figure 14c]. CPF group (2.200 g/kg): compared with model group, spleen trabecular, the red pulp cells, and white pulp cells of mice with normal coloring and recovery in good condition. However, around the posterior artery still gathered a bit of inflammatory cells infiltration [Figure 14d]. CPF group (1.100 g/kg): compared with model group, spleen trabecular, the red pulp cells, and white pulp cells of mice with normal coloring and recovery. However, around the posterior artery, still gathered a few of inflammatory cells infiltration [Figure 14e].

PEF group (0.01233 g/kg): compared with model group, spleen trabecular, the red pulp cells, and white pulp cells of mice with normal coloring and recovery in good condition. However, around the posterior artery gathered a bit of inflammatory cells infiltration [Figure 14f].

PEF group (0.006165 g/kg): compared with model group, spleen trabecular, the red pulp cells, and white pulp cells of mice with normal coloring and improved structure. However, around the posterior artery gathered a lot of inflammatory cells infiltration [Figure 14g].

WEF group (1.964 g/kg): Similar to PEF group (0.01233 g/kg) [Figure 14h].

WEF group (0.982 g/kg): Similar to PEF group (0.006165 g/kg) [Figure 14i].

Histopathological findings in gastric tissues

The histopathological observations of the control group showed regular duplication, no edema, and no expansions in cells [Figure 15a]. However, irregular duplication, cell expansions and edema can be observed in the model group [Figure 15b]. Positive drug group: compared with model group, mice show more regular duplication; intercellular substance of surface mucous cells with almost disappeared expansion and edema, almost convalescent hyperplasia of lamina propria; a few of eosinophils and lymphocytes infiltration; fundic glands arranged closely and regularly; morphology and structure of cell wall-normal; no hyperemia of the gastric mucosa; gastric pits and fundic gland without edema; a few numbers of infiltrating inflammatory cells [Figure 15c]. CPF group (2.200 g/kg): compared with model group, mice have more regular repetition; intercellular substance of surface mucous cells with almost disappeared expansion and edema, almost convalescent hyperplasia of lamina propria; fundic glands arranged closely and regularly; morphology and structure of cell wall-normal; prolapse of gastric mucosa recovery in good condition; gastric pits and fundic gland with slight edema; a few numbers of infiltrating inflammatory cells [Figure 15d]. CPF group (1.100 g/kg): compared with model group, have regular repetition; intercellular substance of surface mucous cells with slight expansion, edema and hyperplasia of lamina propria; fundic glands arranged closely and regularly; morphology and structure of cell wall normal; slight prolapse of gastric mucosa, gastric pits and fundic gland with edema recovery in good condition [Figure 15e]. PEF group (0.01233 g/kg): compared with model group, mice have more regular recurrence; intercellular substance of surface mucous cells with slight expansion, edema and hyperplasia of lamina propria; fundic glands arranged in disorder; capillary with hyperemia phenomenon in the mucosa; fundic glands swelling and muscularis mucosa hyperplasia all recovery in good condition; some infiltrating inflammatory cells [Figure 15f]. PEF group (0.006165 g/kg): compared with model group, have more regular repetition; intercellular substance of surface mucous cells still with expansion, edema, and slight hyperplasia of lamina propria; fundic glands arranged in disorder; gastric mucosa still with slight prolapse and hyperemia, gastric pits and fundic gland with edema recovery in good condition; some infiltrating inflammatory cells [Figure 15g].

WEF group (1.964 g/kg): Similar to PEF group (0.01233 g/kg) [Figure 15h].

WEF group (0.982 g/kg): Similar to PEF group (0.006165 g/kg) [Figure 15i].

Histopathological findings in colon tissues

The observations of colon tissue are given in the Supplementary Materials Figure S12a-i.

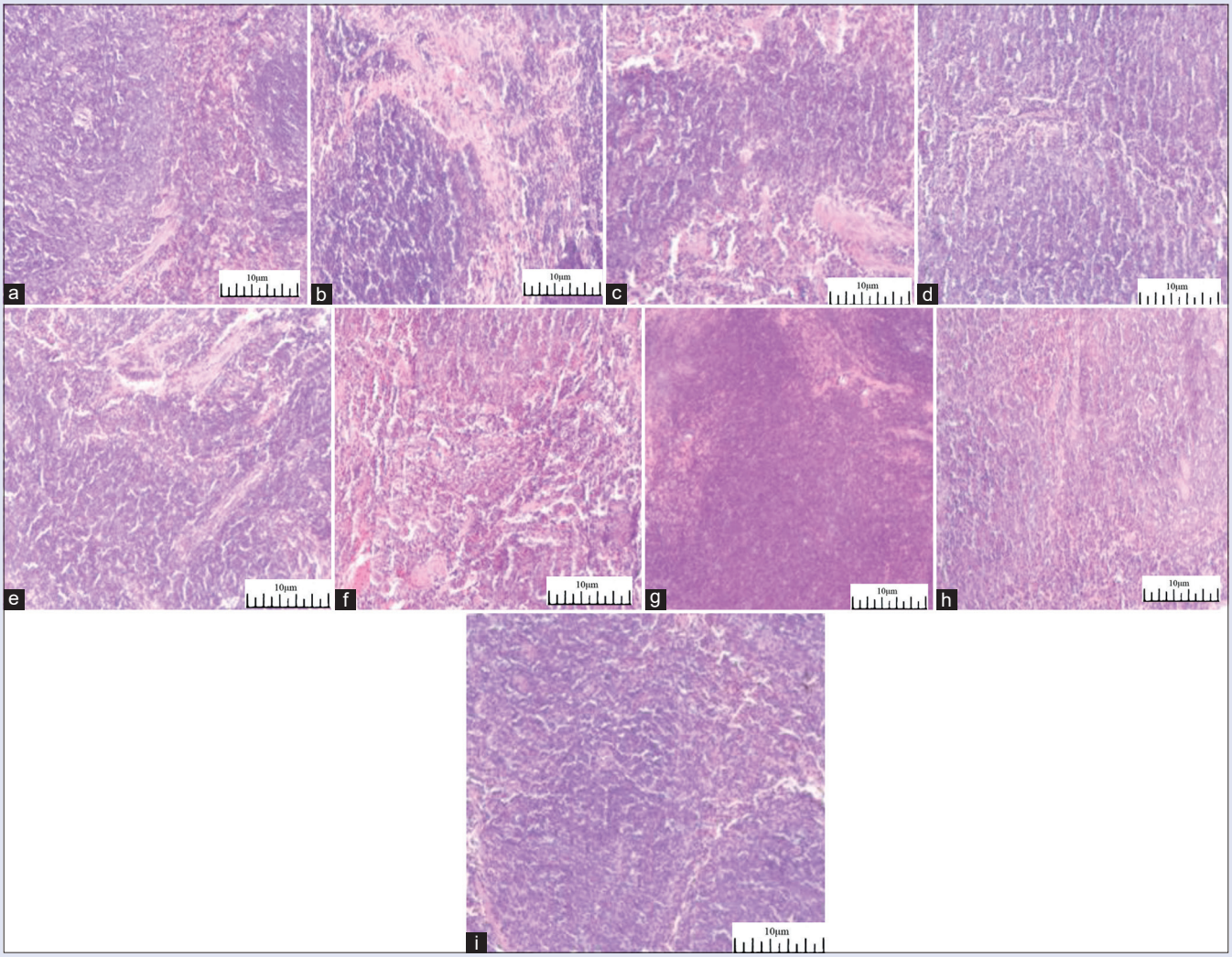

Figure 14: (a-i) Histopathological findings in spleen tissues

DISCUSSION

Drug compatibility is an essential characteristic in the formulation of TCM and pair medicine is always combined to either increase/reinforce functional effects or ameliorate adverse effects. In the present work, the combination principle of AMR/rhubarb pair medicine was explained. Here, an animal model of gastrointestinal diarrhea injury induced by Rhubarb was established to evaluate its alleviating AMR decoction principles and subfractions,[16] *i.e.* WEF, PEF, and CPF. The results showed that the body weight, immune-organ index, D-xylose absorption and intestinal propulsion rates were dramatically increased in the mice administered with water decoction of AMR (6 g/kg). The histopathological findings indicated the protective and healing effect of AMR in spleen functions deficient mice. The AMR fraction treated mice show almost normal cellular structures or cells in the recovering stage. The healing effects of the AMR or its subfractions might be due to polysaccharides and sesquiterpenoids that possessed anti-inflammatory and antioxidant potentials.[17-22]

AMR's water decoction and its five subfractions (WEF, AEF, VOF, PEF, and CPF) were characterized chemically through HPLC analysis. The analysis showed that atractylenolides and polyacetylenes were the primary biomarkers of PEF. Atractylon was found in VOF, 5-hydroxymethyl furfural, and small molecular sugars were detected in WEF, caprolactam, atractyloside A, and polyacetylene acetates were identified in AEF while inulin-type oligosaccharides in CPF.

The gastrointestinal function indicators were decreased in the gastrointestinal injured mice model induced by Rhubarb. After administration of AMR *i.g.*, the injured gastrointestinal functions were recovered, and 6 g/kg of AMR water decoction was discovered to be the most effective dose to treat gastrointestinal injury induced by Rhubarb. The protective effect might be due to the presence of polysaccharides. Polysaccharides regulate the intestinal flora and upregulation of villin expression, which is linked to intestinal injury healing.[23,24] Immunochemical assays indicated that the animal groups with the water decoction and 5 subfractions of AMR (WEF, AEF, VOF, PEF, and CPF) were recovered at different extents as compared with the model group. Besides, the immune functions, intestinal motility, and absorption function of mice were regulated and recovered by the water decoction and WEF, PEF, and CPF of AMR. The presence of atractylenolides, to some extent, is responsible for intestinal protection.[25] Our studies are supported the previous studies on AMR's alcohol extracts, where gastrointestinal function gets improved through the polyamine-Kv1.1 channel signaling pathway and nonmuscle myosin II protein expression in IEC-6 cells.[26,27]

Our results also indicated that different doses of the active components in AMR were administered *i.g.*, for 5 days, gastrointestinal digestion and absorption function were enhanced to varying degrees as compared

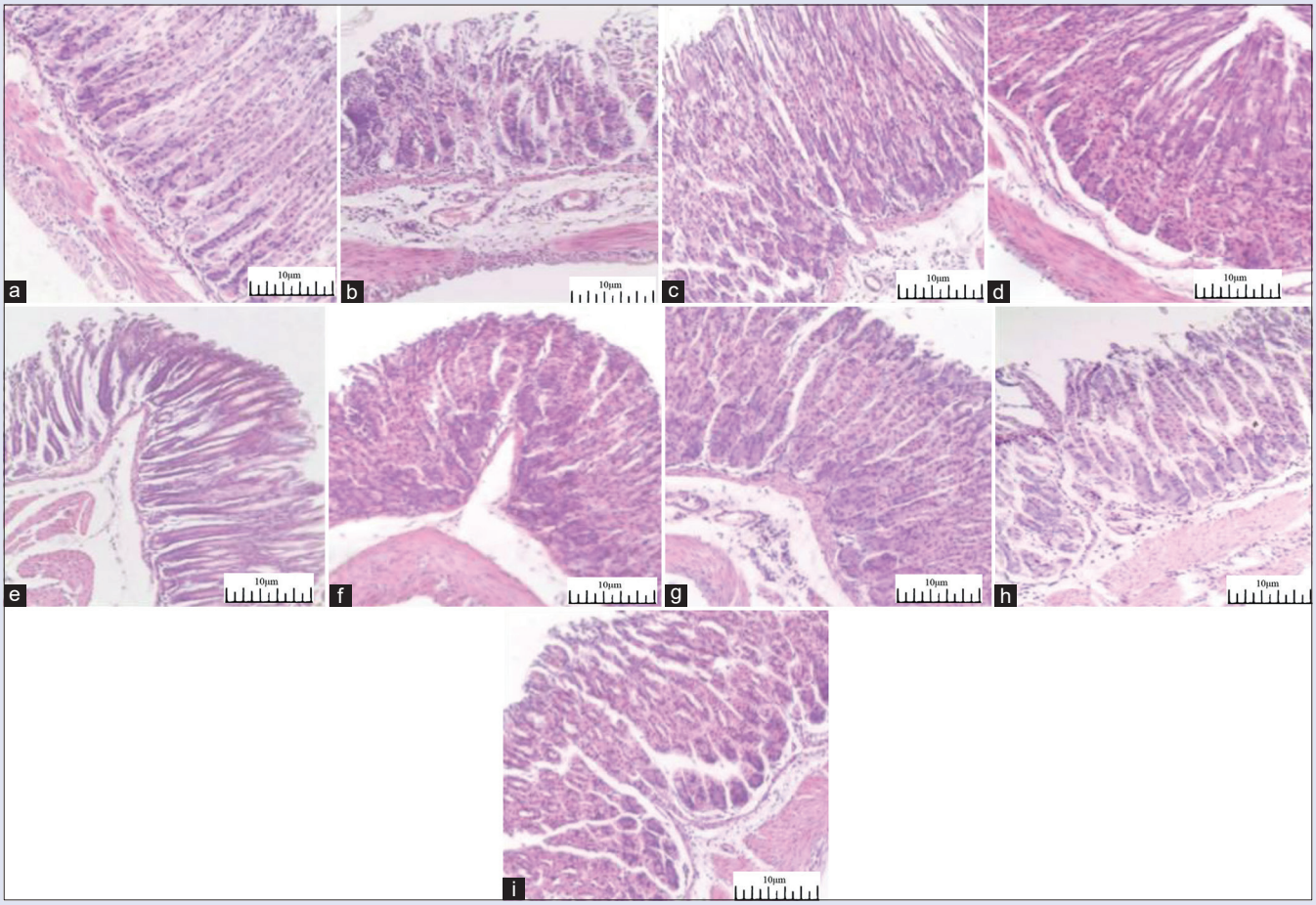

Figure 15: (a-i) Histopathological findings in Gastric tissues

with the model group, the AMS and GAS were dramatically increased in mice administered by WEF, PEF, and CPF like the enhanced salivary amylase in rabbit model system,[28] while VIP was decreased. Significantly, among the effective components of AMR, the CPF was the most efficient fraction. The principal components of AMR and CPF are inulin-type polysaccharides reported to have various functions clinically for the modulation of digestion. Above all, WEF, PEF, and CPF were the most effective components to alleviate diarrhea and gastrointestinal injury induced by Rhubarb and thus may be the effective substances to minimize the adverse effect of Rhubarb.

CONCLUSION

Rhubarb was always used as a model drug for the spleen deficiency in the animal in the Chinese medicine. It can cause diarrhea, etc., which is similar to spleen deficiency symptoms, and clinically, it did possess various side effects and was rarely used alone. For the first time to unravel, AMR, a holy medicine for tonifying spleen, could alleviate the side effects of Rhubarb, and its effective fractions (WEF, PEF, and CPF) were disclosed. Our experiments could reveal the rationality of the pair medicine AMR and Rhubarb and lay a foundation for the elucidation of AMR's effective substance for spleen invigorating.

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Conflicts of interest

There are no conflicts of interest.

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