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RESEARCH ARTICLE

Study on the Acute and Sub-acute Toxicity of Jia Wei San Huang Tang in Mice and Rats

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A B S T R A C T

Jia Wei San Huang Tang (JWSHT) alleviates the cold properties of the original formula, San Huang Tang, and holds promise for treating gastrointestinal ailments. The primary objective of this study was to validate the safety of the JWSHT in a rat model and position the experimental foundation for future comprehensive investigations into its pharmacological effects and safe clinical application. Guided by the "Methodology of Pharmacological Research on Traditional Chinese Medicine," JWSHT underwent oral toxicity assessments. In the acute study, 60 Kunming mice (half male, half female) were categorized into five groups, receiving gavage doses of 16.0, 12.8, 10.2, and 8.2g/kg JWSHT for 7 days. Symptoms and mortality were recorded, and LD50 was calculated. To ascertain the maximum tolerated dose, mice received 128g/kg of the product and were observed for 14 days. In the subacute rat study, 80 rats were divided into three treatment groups and one control, administered doses of 16g/kg/d, 8g/kg/d, and 4g/kg/d for 30 days. Subsequently, rats were euthanized, and diverse parameters were analyzed to evaluate JWSHT's subacute toxicity. The acute toxicity test revealed that the LD50 was greater than 5g/kg. No signs of toxicity were observed in mice when administered at the maximum dose. The results of the subacute toxicity test indicated that the hemoglobin (HGB) levels in the high-dose group and the mean platelet volume (MPV) in the low-dose group were significantly higher than those in the control group ($P<0.05$). The alanine aminotransferase (ALT) levels in the low-dose group were significantly lower than the control group ($P<0.05$). The relative spleen weight in the male high-dose group was significantly higher than that in the control group ($P<0.05$). Mild bleeding was observed in the kidneys of the high-dose group, while other parameters showed no significant difference compared to the control group ($P>0.05$). Therefore, it was concuded that under the conditions of this study, the administration of JWSHT was relatively safe.

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INTRODUCTION

Chinese herbal medicine has a history of thousand years in the field of medicine, characterized by the synergistic effects of multiple components. By skillfully combining various herbal ingredients through precise proportions and administration methods, these formulations have demonstrated outstanding therapeutic efficacy (Lee *et al*., 2022; Li *et al*., 2022). Recorded in the Ming Dynasty's "Pocket Prescriptions," San Huang Tang (SHT) is a formula, primarily composed of *Coptis*, *Scutellaria baicalensis*, and *Phellodendron amurense*. SHT holds a significant position in the treatment of gastrointestinal diseases, particularly in addressing damp-heat diarrhea (Wang *et al*., 2014; Yin *et al*., 2021; Meng *et al*., 2022). Its historical application dates back centuries, and it continues to be widely used, providing effective treatment and relief to numerous patients. Although the effectiveness of classic herbal formulas is widely acknowledged, concerns about their safety arise when medical researchers continually modify these formulations, especially when introducing new herbal components, in pursuit of improved therapeutic outcomes and reduced side effects (Zhu *et al*., 2019; Cheng *et al*., 2022).

Through its mechanisms of anti-inflammatory, antibacterial, immunomodulatory and antioxidant actions,

SHT can alleviate various diseases such as diabetes, fatty liver, enteritis and cancer (Wu *et al*., 2019; Zhao *et al*., 2019; Chen *et al*., 2022; Wang *et al*., 2022). Additionally, it also effectively regulates the internal environment of the human body through exerting positive regulatory effects upon key physiological processes such as cellular metabolism and immune responses (Zhao *et al*., 2019; Tawulie *et al*., 2023). However, traditional SHT, due partly to its strong bitter taste and cold properties (Jin *et al*., 1995), may lead to potential side effects and discomfort with prolonged use, particularly affecting the digestive function, thus resulting in symptoms such as loss of appetite, abdominal distension, nausea, and vomiting. To optimize the efficacy of SHT and reduce its potential side effects, the original SHT formula was augmented with Chinese traditional medicinal herbs, *Atractylodes Lancea*, *Fried Astragalus membranaceus*, *Prepared licorice*, and *Divine comedy* to form a modified SHT as Jia Wei SHT (JWSHT). This purpose was to harmonize the properties of the SHT and potentially introduce new therapeutic effects. Literature shows that *Astragalus membranaceus* and *Prepared licorice* have been proven to enhance immunomodulation and antioxidant effects (Leite *et al*., 2022; Chen *et al*., 2020), while the inclusion of *Divine comedy* and *Atractylodes Lancea* can balance the strong cold nature of SHT, thereby reducing its potential harm to the spleen and stomach (Qu *et al*., 2022; Liu *et al*., 2023). The goal of the formula of JWSHT is to construct a more comprehensive and effective treatment regimen that is also safer for patients. However, JWSHT is more complex in both its composition and effects, and this complexity might introduce new safety concerns. Therefore, to ensure the safe clinical application of JWSHT, this study utilized rat and mouse models to evaluate the toxicity levels of JWSHT, providing scientific evidence for its safety.

MATERIALS AND METHODS

Source of medicinal materials: *Coptis* (Dried rhizome of plants from the *Ranunculaceae* family, Sichuan, Batch No.: 22012503), *Phellodendron amurense* (Dried bark of the *Phellodendron* tree from the *Rutaceae* family, Anhui, Batch No.: 22040201), *Scutellaria baicalensis* (From the *Lamiaceae* family, Shanxi, Batch No.: 220105003), *Atractylodes Lancea* (Rhizome of the *Atractylodes* from the *Asteraceae* family, Anhui, Batch No.: 2112268), *Fried Astragalus membranaceus* (Roasted root of Astragalus from the *Fabaceae* family, Inner Mongolia, Batch No.: J210301), *Prepared licorice* (Processed dried root and rhizome of licorice from the *Fabaceae* family, Inner Mongolia, Batch No.: 211102), and *Medicated leaven* (A fermented mixture of *Polygonum hydropiper*, *Artemisia annua*, *almond mud*, *adzuki beans*, and fresh *Atractylodes ear grass* added to flour or bran, Sichuan, Batch No.: 202006082) were purchased from Tong Ren Tang pharmacy in Urumqi, Xinjiang, China. The botanical materials were morphologically identified in our laboratory and met the quality standards of the "Chinese Pharmacopoeia" (IPC, 2015).

Preparation and identification of JWSHT: According to the "Chinese Pharmacopoeia" (IPC, 2015), *Coptis*, *Phellodendron amurense*, *Scutellaria baicalensis*, *Atractylodes Lancea*, *Fried Astragalus membranaceus*, *Prepared licorice*, and *Divine comedy* were ground into powder in a ratio of 10:10:10:15:15:6:10. The medicinal materials were soaked in distilled water at a 10 weight/volume (1:10, w/v) ratio for 30 minutes, then cooked for 2 hours and extracted twice. The filtrates were combined and concentrated to a raw medicine concentration of 1.6g/mL. For the experiments, the medicinal solution was diluted with distilled water to the desired concentration.

An appropriate amount of the powdered medicinal sample was weighed and added to 1 mL of 80% methanol, followed by ultrasonication for 10 minutes. It was then centrifuged at 14,000 rpm for 10 minutes. 0.8 mL of the supernatant was transferred to a centrifuge tube and centrifuged again. The resulting supernatant was placed into a sample vial. With a column temperature of 35°C and a flow rate of 0.2mL/min, the sample was separated on a chromatographic column. The separated compounds were ionized and introduced into a mass spectrometer. Subsequent mass spectrometric data collection was performed using the Q Exactive Plus Orbitrap (Thermo Fisher, Waltham, MA, USA) high-resolution mass spectrometer. Both positive and negative ion modes were scanned simultaneously, with a scan range of m/z 100-1200. MS1 resolution was set to 70,000 and MS2 resolution was set to 17,500. The ion source voltage was 3.2kV, the capillary ion transfer tube temperature (Capillary temp) was 320°C, the auxiliary gas heating temperature (Aux gas heater temp) was 350°C, the sheath gas flow rate was 40L/min and the auxiliary gas flow rate was 15L/min. The AGC Target was set to 1e6, and the TopN was set to 5. The collision energy triggering MS2 scanning used a stepped fragmentation voltage NCE, set at 30, 40 and 50. Analysis was conducted using Compound Discoverer 3.3 software. Identification of each component was achieved by comparing the retention time, molecular weight (mass deviation <10 ppm) and MS2 fragment ions with the metabolites in the local mzVault database.

Animals and ethics: In this study, we utilized healthy Kunming (KM) mice, approximately 5 weeks old with a weight of around 20±2 g (both males and females, n = 100), and Sprague-Dawley (SD) rats, approximately 7 weeks old with a weight of around 200±20 g (both males and females, n = 80). The animals were sourced from the Animal Center of Xinjiang Medical University (2017013). They were housed in a room with a 12-hour artificial light cycle, at a temperature of 23±2°C, and a humidity of 50-65%. The animals were fed a standard diet and underwent an acclimatization period of 1 week. All animal experiments were conducted in accordance with ethical standards and were approved by the Ethics Committee of Xinjiang Agricultural University.

Acute oral toxicity study

Determination of median lethal dose (LD50): Based on the preliminary experimental results, the Hodge and Sterner method was employed. Sixty mice were randomly divided into five groups, with six mice in each group, evenly split between males and females. The mice were

fasted for 12 hours prior to dosing (without water deprivation) and were administered doses of 16.0, 12.8, 10.2, and 8.2g/kg via oral gavage. The control group received an equivalent volume of physiological saline. Observations were made continuously for 7 days, with checks conducted once in the morning and once in the afternoon, meticulously recording the mice's body weight, toxic reactions, and mortality. The LD50 was calculated using the modified Karber's method formula: LD50 = $lg^{-1}[Xm - i(\Sigma P - 0.5)]$, where Xm was the logarithmic value of the dose in the highest dose group; was the logarithmic value of the dose ratio; and ΣP was the sum of the mortality rates across all groups. If any mice died, a post-mortem examination was conducted. If no deaths occurred and further dosing was not feasible, a maximum dose test was performed.

Determination of maximum dosage: Forty mice were evenly divided into two groups, with 20 mice in each group, half male and half female. The mice were fasted for 12 hours but allowed access to water. Mice in the experimental group were administered the herbal compound at the Maximum Tolerated Dose Group (MTDG) (maximum permissible concentration of 1.6g/mL, 0.8mL per administration, dosed twice within 24 hours), while the control group received an equivalent volume of physiological saline. After oral administration, the mice were routinely housed for 14 days. Daily observations were made on the mice's mental state, and records were kept on their body weight, symptoms of poisoning, mortality rate, and time of death.

Sub-acute toxicity study: Based on the "Methodology of Pharmacological Research on Traditional Chinese Medicine"(Qi. 2006), the low dose in the subacute toxicity test was designed with reference to the clinical dose. The recommended dose of JWSHT for humans was 0.65g/kg. When converted to the rat dosage based on body surface area, it was approximately 4g/kg. Therefore, 4g/kg was chosen as the lowest administering dose for the subacute toxicity test. Subsequently, using the Hodge and Sterner method, SD rats, both female and male were divided into four groups, with 20 rats in each group (10 females and 10 males): High Dose Group (HDG) (16g/kg), Middle Dose Group (MDG) (8g/kg), Low Dose Group (LDG) (4g/kg), and Control Group (CG). Animals in each group were administered once daily at the allocated dose, with a gavage dose of 20mL/kg, continuously for 30 days. Daily records were kept on general behavior, clinical toxicity, mortality rate, and body weight. Cumulative weight gain (%) was calculated based on the initial weight. At the end of the dosing period, SD rats were fasted for 12 hours. Blood was drawn from the abdominal aorta. The rats were euthanized using an excessive amount of pentobarbital sodium, and various organs (heart, liver, spleen, lungs, kidneys, stomach, duodenum) were excised and weighed.

Hematological analysis: Blood was collected into anticoagulant tubes and analyzed using the ZC-980 Hematology Analyzer (Jilin Zichen Photoelectricity Technology Co., Ltd.).

Serum biochemistry analysis: Blood was collected into anticoagulant tubes and centrifuged at 1000 rpm for 3 minutes. Extract serum and place it into the Catalyst biochemical test kit (Adex Maine Bioproducts Trade Co., Ltd.). And used the PointcareM4 biochemical analyzer (Tianjin Mnchip Technology Co., Ltd.) to test four indicators: Creatinine (CREN), Blood urea nitrogen (BUN), Alanine Transaminase (ALT) and Aspartate Aminotransferase (AST).

Histopathological examinations: After weighing, the major organs (liver, heart, spleen, lungs, kidneys, stomach, and duodenum) from each group were immediately fixed in 4% formaldehyde (Gansu Weiboxin Biotechnology Co., Ltd.). After 24 hours, the tissues were dehydrated, embedded in paraffin, and sectioned into 4-5μm thick slices. The sections were then stained with Hematoxylin and Eosin (H&E) (Gansu Weiboxin Biotechnology Co., Ltd.) and observed under an IX53 inverted microscope (Olympus Corporation Co., Ltd, Japan) (Martey *et al*., 2010; Afolabi *et al*., 2012).

Statistical analysis: Data were presented as mean ± standard error of the mean (SEM). Statistical comparisons of the data were performed using the Statistical Package for the Social Sciences v26.0 for Windows (SPSS Inc., Chicago, IL, USA), including one-way or two-way analysis of variance, followed by a t-test to assess differences between groups. A P-value of less than 0.05 was considered statistically significant.

RESULTS

HPLC-MC analysis: The total ion chromatograms of JWSHT were generated using the Q Active Orbitrap high-resolution mass spectrometer in both positive and negative ion modes. Upon screening and analyzing the detected compounds, it was found that the modified San Huang Tang primarily contained 175 chemical components. These included flavonoids (60/175, 34%), alkaloids (25/175, 14%), acids (23/175, 13%), esters (11/175, 6%), terpenes (6/175, 3%), sugars (5/175, 3%), saponins (3/175, 2%), and some other types of compounds (Fig. 1, Table 1).

Acute oral toxicity

LD50 of JWSHT: After oral administration, two mice in the 12.8g/kg group exhibited symptoms of lethargy and disheveled fur immediately after gavage, but they recovered to their normal state after 6 hours. The rest of the mice appeared to be in good spirits and showed no adverse reactions. Continuous observation for 7 days revealed no abnormal reactions in any of the mice. There was no statistically significant difference in weight between the groups ($P > 0.05$). Upon dissection, no abnormal pathological changes were observed in the major organs by the naked eye. The LD50 in this experiment was found to be greater than 5g/kg. According to toxicological evaluation standards and drug toxicity grading criteria (OECD, 2002), when the LD50 is greater than 5g/kg, the drug can be considered non-toxic.

Table 1: Chemical constituents of Jiawei San Huang Tang

Name	Formula	Annot. DeltaMass [ppm]	Calc. MW	m/z	RT [min]	mzVault Best Match	Reference Ion	Group Area:2	Group Area:1
Berberine	C20 H17 N O4	-1.02	335.1154	336.1227	24.566	96.7	[M+H]+1	27048954291	17936393688
								4.85	8.61
Palmatine	C21 H21 N O4	-0.43	351.1469	352.1542	24.396	90	[M+H]+1	63787871398	52133013692
Coptisine chloride			319.0843	320.0916	23.361	89.9	[M+H]+1	51798087523	42738704700
Baicalin	C21 H18 O11	-0.25	446.0848	447.0921	24.021	89.3	[M+H]+1	33809791432	33225869603
Epiberberine	C20 H17 N O4	-0.97	335.1154	336.1227	23.265	91	[M+H]+1	29503932560	28830114155
Wogonoside	C22 H20 O11	-0.23	460.1005	461.1077	25.934	83.1	[M+H]+1	24656245072	28566871237
Jatrorrhizine	C20 H19 N O4	-1.02	337.1311	338.1383	23.17	94.9	[M+H]+1	24408586193	21539816085
(+)-Magnoflorine	C20 H23 N O4	-0.33	341.1626	342.1699	20.12	92.6	[M+H]+1	22462696670	17839431577
Phellodendrine chloride			341.1626	342.1699	19.543	87.8	[M+H]+1	20029661970	11959126242
Berberrubine	C19 H15 N O4	-0.35	321.1	322.1073	22.212	83.5	[M+H]+1	9885072298	8918333879
Wogonin	C16 H12 O5	-0.64	284.0683	285.0755	31.963	89.2	[M+H]+1	8164652843	11802509959
Baicalein	C15 H10 O5	-0.5	270.0527	269.0455	28.533	89.7	[M-H]-1	7564244617	11336697169
2-Pyrrolidinecarboxylic acid L-	C5 H9 N O2	0.16	115.0634	116.0706	1.554	81.4	[M+H]+1	7235441419	6204751550
Oroxylin A-7-O-β-D-glucuronide	C22 H20 O11	-0.22	460.1005	461.1077	25.348	84	[M+H]+1	6847516745	7351214728
4-Methylumbelliferone 7	C10 H8 O3	-0.65	176.0472	209.0807	19.608	81.1	[M+H+MeOH]+1	5846780531	
Sucrose	C12 H22 O11	-0.49	342.1161	341.1087	1.563	95.2	[M-H]-1	5734991496	5648860698
Genipin 1-O-β-D-gentiobioside	C23 H34 O15	-0.21	550.1897	595.1879	18.746	92.6	[M+FA-H]-1	5372033696	
Citric acid	C6 H8 O7	-0.27	192.027	191.0197	1.667	90.3	[M-H]-1	4637050242	2901982678
Chrysosplenetin B	C19 H18 O8	-0.59	374.1	375.1072	32.344	70.6	[M+H]+1	4038140086	5762896060
Liquiritin	C21 H22 O9	0.11	418.1264	417.1192	21.725	87.2	[M-H]-1	3343108464	3295383608
Gardenoside	C17 H24 O11	-0.32	404.1317	449.1299	17.521	94.6	[M+FA-H]-1	3251411077	
Isoliquiritigenin	C15 H12 O4	-0.58	256.0734	257.0807	21.723	91.3	[M+H]+1	2895003511	2649853485
Oxoglaucine	C20 H17 N O5	-0.07	351.1107	352.1179	22.476	82.1	[M+H]+1	2791446081	2056453278
Chlorogenic acid	C16 H18 O9	-0.42	354.0949	353.0877	19.045	93.2	[M-H]-1	2475604548	1815587361
Diammonium glycyrrhizinate	C42 H62 O16	-0.19	822.4036	823.4104	30.253	91.8	[M+H]+1	2198270174	1743600045
Liguiritigenin-7-O-β-D-apiosyl-4'-O-β-D-glucoside	C26 H30 O13	-0.2	550.1685	549.1613	21.549	85.1	[M-H]-1	2186304967	1754155883
Oroxylin A	C16 H12 O5	-0.69	284.0683	285.0755	32.761	86.9	[M+H]+1	2168268829	3123126262
Glycyrrhizic acid	C42 H62 O16	-0.01	822.4038	821.3966	31.22	93.3	[M-H]-1	1916467793	1278957722
18 β-Glycyrrhetintic Acid	C30 H46 O4	-0.55	470.3394	453.3361	31.221	84.5	[M+H-H2O]+1	1680769553	1254210380
Quinic acid	C7 H12 O6	-0.58	192.0633	191.056	20.673	90.4	[M-H]-1	1483898288	1205147638
Demethyleneberberine	C19 H17 N O4	-0.91	323.1155	324.1227	21.879	84.5	[M+H]+1	1219187093	1375367973
Shanzhiside	C16 H24 O11	0.06	392.1319	391.1246	16.827	94.7	[M-H]-1	1123134865	
Trigonelline HCl	C7 H7 N O2	0.19	137.0477	138.055	1.558	89.3	[M+H]+1	1109410725	888224313.8
Taxifolin	C15 H12 O7	-0.1	304.0583	303.051	20.931	85.2	[M-H]-1	1049044418	1729196170
7-Methoxycoumarin	C10 H8 O3	-0.66	176.0472	209.0807	18.746	83.5	[M+H+MeOH]+1	1027608800	
Dihydropalmatine	C21 H23 N O4	0.09	353.1627	354.17	22.581	76.5	[M+H]+1	1020975116	714134487.2
Manninotriose	C18 H32 O16	0.11	504.1691	549.1673	2.424	85	[M+FA-H]-1	946841215.6	679558877.1
Caffeic acid	C9 H8 O4	-0.36	180.0422	163.0389	19.061	78.3	[M+H-H2O]+1	945005428	681077120.6
Geniposide	C17 H24 O10	-0.49	388.1368	387.1296	19.605	90.1	[M-H]-1	943655172.4	10026172.01
L-Leucine L-	C6 H13 N O2	0.71	131.0947	132.102	4.101	71.3	[M+H]+1	938051582.2	1079188322
L-Glutamic acid L-	C5 H9 N O4	-0.14	147.0531	295.1135	1.463	81.1	[2M+H]+1	886297461	808632738.2
Cryptochlorogenic acid	C16 H18 O9	-0.4	354.0949	353.0877	17.659	89.1	[M-H]-1	885928381.6	360122304.8
Glabrolide	C30 H44 O4	-0.03	468.324	469.3312	27.55	76	[M+H]+1	827469382.6	665288845.8
Eriodictyol	C15 H12 O6	-0.77	288.0632	271.0599	24.884	84	[M+H-H2O]+1	746077057.1	777307737.4
Calycosin-7-O-β-D-glucoside	C22 H22 O10	-0.54	446.1211	447.1283	21.504	81.8	[M+H]+1	664121966.3	467399260.1
Limonin	C26 H30 O8	-0.07	470.194	515.1922	31.014	84	[M+FA-H]-1	662305995.9	767305049.5
5-Hydroxymethylfurfural 5-	C6 H6 O3	-0.08	126.0317	127.039	1.572	76.8	[M+H]+1	605782900.5	640474120.3
Formononetin	C16 H12 O4	-0.17	268.0735	269.0808	29.656	88.2	[M+H]+1	598112212.8	538469631.8
Chrysin	C15 H10 O4	-0.24	254.0579	253.0506	32.203	91	[M-H]-1	581269945.4	756359267.5
Hydroxygenkwanin	C16 H12 O6	-1.06	300.0631	301.0703	25.036	71	[M+H]+1	575155484.1	779170624.9
Isoferulic acid	C10 H10 O4	0.23	194.058	195.0653	20.562	87.4	[M+H]+1	562985232.8	375178346
Dehydrocorydaline	C22 H23 N O4	-0.59	365.1625	366.1698	25.381	88.6	[M+H]+1	561603631.6	876104689.3
Shikimic acid	C7 H10 O5	-1.54	174.0526	173.0453	20.529	79	[M-H]-1	524411878	377858590
Isoguanosine	C10 H13 N5 O5	-0.04	283.0917	282.0844	11.714	85.2	[M-H]-1	513943533.4	
Tetrahydropalmatine HCl	C21 H25 N O4	-0.68	355.1781	356.1854	22.17	73.9	[M+H]+1	497526284.6	227440095.8
Stachydrine	C7 H13 N O2	0.12	143.0947	144.1019	1.563	70	[M+H]+1	490994402.7	411619601.6
Scutellarin	C21 H18 O12	0.31	462.08	463.0872	21.991	85.9	[M+H]+1	477738701.8	453756349.9
Liquiritigenin	C15 H12 O4	-0.09	256.0735	255.0663	25.195	82.2	[M-H]-1	470875444.2	498276154.3
Quillaic acid	C30 H46 O5	-0.16	486.3344	469.3312	29.1	81.8	[M+H-H2O]+1	470663275.5	264980741
Tetrahydropalmatine	C21 H25 N O4	-0.68	355.1781	356.1854	20.914	71.6	[M+H]+1	463273825.7	355378410.5
Rutin	C27 H30 O16	-0.38	610.1532	609.1458	21.508	90.2	[M-H]-1	456659454.2	6345297.347
p-Coumaric acid	C9 H8 O3	0.74	164.0475	182.0813	4.912	83.6	[M+NH4]+1	455077115.9	470759340.1
Stachyose	C24 H42 O21	0.12	666.2219	665.2147	1.582	91.3	[M-H]-1	449038794.1	546752959.8
Morin	C15 H10 O7	-0.02	302.0427	301.0354	22.235	81.5	[M-H]-1	391442269.5	574739441.7
Crocetin	C20 H24 O4	-0.07	328.1674	329.1747	23.225	81.6	[M+H]+1	389005369.7	
Uridine	C9 H12 N2 O6	-0.03	244.0695	243.0622	5.076	92.7	[M-H]-1	332445532.1	356751132.6
Puerarin	C21 H20 O9	0.25	416.1108	415.1036	22.765	78.4	[M-H]-1	326208123	447255766.2
Calycosin	C16 H12 O5	-0.23	284.0684	283.0612	22.872	90.1	[M-H]-1	314381652.7	460362314.4
Nicotinic acid	C6 H5 N O2	0.85	123.0321	124.0394	2.644	82	[M+H]+1	284312516.2	362011207.2
Lawsone	C10 H6 O3	-0.1	174.0317	207.0652	23.707	71.3	[M+H+MeOH]+1	281651795.8	

Azelaic acid	C9 H16 O4	-0.3	188.1048	187.0975	23.145	84.2	[M-H]-1	278085475	300618197.5
Lysionotin	C18 H16 O7	-0.25	344.0895	345.0968	33.594	70.7	[M+H]+1	262076754.9	398114821.2
Nicotinamide	C6 H6 N2 O	0.28	122.0481	123.0553	3.06	79.2	[M+H]+1	246554136.1	391078122.4
Danshensu	C9 H10 O5	-0.52	198.0527	197.0455	16.109	90.1	[M-H]-1	240787784.4	680010015.7
Citropten	C11 H10 O4	-0.07	206.0579	207.0652	22.11	79.8	[M+H]+1	237930222.2	
Geniposidic acid	C16 H22 O10	-0.1	374.1213	373.114	17.06	94.1	[M-H]-1	221689420.5	
Maltopentaose	C30 H52 O26	-0.03	828.2747	827.2674	1.586	88.7	[M-H]-1	197693522	165539139.9
Dehydrocostus lactone	C15 H18 O2	0.09	230.1307	231.138	33.477	84.3	[M+H]+1	191619291.5	220007612.3
Hispidulin	C16 H12 O6	-0.92	300.0631	301.0703	29.182	81.2	[M+H]+1	172948812.6	442303372.2
Iristectorigenin B	C17 H14 O7	-0.13	330.0739	331.0812	28.272	78	[M+H]+1	172587440.9	254596632.2
Atractylenolide II	C15 H20 O2	-0.29	232.1463	233.1535	37.73	89	[M+H]+1	162189254.6	44729794.37
4-Methyl-6,7-dihydroxycoumari	C10 H8 O4	-0.51	192.0422	225.0756	17.527	70.6	[M+H+MeOH]+1	161940500.8	
7-Methoxy-4-methylcoumarin 7-	C11 H10 O3	-0.82	190.0628	191.0701	20.712	81.1	[M+H]+1	161683358.1	
Icaritin	C21 H20 O6	-0.05	368.126	367.1187	33.261	77.6	[M-H]-1	161297412.8	417463495
α-Linolenic acid α-	C18 H30 O2	-0.01	278.2246	279.2319	40.214	79.2	[M+H]+1	156275018.4	116332071.7
Salicylic acid	C7 H6 O3	-0.85	138.0316	137.0243	23.352	78	[M-H]-1	153535055.8	163314826.9
Cytosine	C4 H5 N3 O	0.02	111.0433	112.0505	2.698	84	[M+H]+1	147083023.7	153315462.5
Isoliquiritin	C21 H22 O9	0.24	418.1265	417.1192	24.098	88.7	[M-H]-1	133221996.5	138079214.6
Naringenin	C15 H12 O5	-0.64	272.0683	273.0756	22.996	88.3	[M+H]+1	130624336.2	117904472.3
Luteolin	C15 H10 O6	-0.34	286.0476	287.0549	23.923	80.3	[M+H]+1	109971069.5	174778031.7
Adenine	C5 H5 N5	-0.34	135.0545	134.0472	2.712	76.2	[M-H]-1	103489155.5	99665421.46
Orcinol gentiobioside	C19 H28 O12	0.09	448.1581	447.1508	16.925	77.3	[M-H]-1	98328507.74	84109063.83
4-Methoxysalicylic acid 4-	C8 H8 O4	-0.95	168.0421	167.0348	17.063	75.3	[M-H]-1	96150418.34	64191364.66
2-Hydroxy-4-methoxybenzaldehyde 4-	C8 H8 O3	0.34	152.0474	153.0547	21.031	83	[M+H]+1	93414750.07	74368031.55
Naringenin chalcone	C15 H12 O5	-0.65	272.0683	273.0756	21.609	87.2	[M+H]+1	92071848.33	52729117.94
Aurantio-obtusin	C17 H14 O7	-0.15	330.0739	331.0812	26.767	75	[M+H]+1	91773643.82	126687175.2
Protocatechualdehyde	C7 H6 O3	-0.83	138.0316	137.0243	17.846	86.5	[M-H]-1	89921778.39	83033068.2
3,5-Dicaffeoylquinic acid	C25 H24 O12	-0.05	516.1268	515.1195	22.67	90.2	[M-H]-1	88697066.75	35552088.02
Coumarin	C9 H6 O2	0.08	146.0368	147.0441	20.256	74.5	[M+H]+1	86443372.34	80413423.71
Isoscopoletin	C10 H8 O4	0.05	192.0423	193.0496	19.094	76.1	[M+H]+1	84536549.74	
Astragaloside III	C41 H68 O14	0.47	784.4613	829.4595	29.566	92.2	[M+FA-H]-1	79628037.82	52993130.82
Protocatechuic acid	C7 H6 O4	-0.59	154.0265	153.0192	16.052	87	[M-H]-1	76692878.06	60771807.53
Sophoricoside	C21 H20 O10	0.26	432.1058	433.113	25.794	81.3	[M+H]+1	75004469.4	62533687.67
6''-O-Acetylglycitin 6''-O-	C24 H24 O11	0.76	488.1322	489.1395	23.855	82.4	[M+H]+1	72476473.35	68973408.71
Dehydroglaucine	C21 H23 N O4	0.04	353.1627	354.17	23.057	70.3	[M+H]+1	71450813.55	70224355.61
Isomucronulatol 7-O-glucoside	C23 H28 O10	0.33	464.1684	463.1612	25.121	86.1	[M-H]-1	69788361.94	70746902.5
Aurantio-obtusin β-D-glucoside	C23 H24 O12	0.3	492.1269	493.1342	22.553	76.7	[M+H]+1	69407296.56	99468240.84
L-Tryptophan L-	C11 H12 N2 O2	-0.35	204.0898	203.0826	17.513	81.4	[M-H]-1	69369784.21	85898912.86
Wilforlide A	C30 H46 O3	2.58	454.3459	455.3531	34.117	74.5	[M+H]+1	68022565.49	57997240.36
Sibiricose A5	C22 H30 O14	-0.23	518.1634	517.1561	19.869	86.6	[M-H]-1	65402334.62	77815112.77
Astragalin	C21 H20 O11	0.2	448.1007	447.0934	22.755	72.9	[M-H]-1	62859237.67	21691640.5
Obacunone	C26 H30 O7	0.13	454.1992	455.2065	34.592	81.3	[M+H]+1	60906559.58	88890122.13
Atractylenolide III	C15 H20 O3	-0.08	248.1412	249.1485	33.475	71.7	[M+H]+1	56906183.47	64694686.67
3,4-Dihydroxyphenylethanol	C8 H10 O3	-0.72	154.0629	153.0556	17.107	83.6	[M-H]-1	55363545.41	36704405.63
Isoalantolactone	C15 H20 O2	0.1	232.1464	233.1536	17.904	74.4	[M+H]+1	55067911.68	82064542.7
Emodin-8-O-β-D-glucopyranoside	C21 H20 O10	-0.19	432.1056	431.0982	25.205	81.6	[M-H]-1	53687754.56	79498946.77
p-Hydroxybenzaldehyde	C7 H6 O2	-0.93	122.0367	121.0294	19.74	71.6	[M-H]-1	50820559.04	64381018.63
Sinapic acid	C11 H12 O5	1.41	224.0688	225.0757	22.987	85.4	[M+H]+1	47498693.29	2115314.924
Oxyberberine	C20 H17 N O5	-0.57	351.1105	352.1178	30.99	83.7	[M+H]+1	44266943.49	37221692.3
Retrochalcone	C16 H14 O4	-0.31	270.0891	271.0964	27.459	75.5	[M+H]+1	43954859.8	36418976
Iridin	C24 H26 O13	0.17	522.1374	521.1302	23.01	77.2	[M-H]-1	41589249.35	64903825.96
Kaempferol	C15 H10 O6	-0.73	286.0475	287.0548	20.978	73.3	[M+H]+1	40749305.51	32626254.75
Higenamine	C16 H17 N O3	-0.57	271.1207	272.128	18.037	78.4	[M+H]+1	39851995.13	30705383.55
Verbascoside	C29 H36 O15	0.18	624.2055	623.1983	21.599	90.7	[M-H]-1	39803249.19	129856190.8
Licochalcone B	C16 H14 O5	-0.65	286.0839	285.0767	24.828	77.9	[M-H]-1	39680046.57	29486888.52
Dictamnine	C12 H9 N O2	0.12	199.0634	200.0706	29.588	70.1	[M+H]+1	37724673.2	30169953.83
Grosvenorine	C33 H40 O19	0.7	740.2169	739.2096	21.035	85.5	[M-H]-1	37555299.08	
Pinocembrin	C15 H12 O4	-0.07	256.0735	255.0663	32.482	85.4	[M-H]-1	34186278.5	43214161.16
Arglabin	C15 H18 O3	-0.13	246.1256	247.1328	29.249	74.2	[M+H]+1	33708994.45	37932066.06
Crocin II	C38 H54 O19	0.83	814.3266	859.3247	28.254	83.5	[M+FA-H]-1	30802562.89	
Loganic acid	C16 H24 O10	-0.27	376.1369	375.1296	17.597	70.5	[M-H]-1	30639261.02	6654104.213
Vicenin II	C27 H30 O15	0.22	594.1586	595.1657	19.774	79.1	[M+H]+1	30225338.05	25469962.06
Parthenolide	C15 H20 O3	-0.15	248.1412	249.1485	26.807	81.7	[M+H]+1	29968185.43	29016137.84
5,7,3'-Trihydroxy-6,4',5'-trimethoxyflavone 5,7,3'-	C18 H16 O8	-0.61	360.0843	361.0915	29.224	86	[M+H]+1	29264583.79	
Gentisic acid	C7 H6 O4	-0.75	154.0265	153.0192	18.203	87.9	[M-H]-1	29079702.89	24872015.23
Salidroside	C14 H20 O7	-0.07	300.1209	345.1191	17.918	84.6	[M+FA-H]-1	28920199.62	16641012.27
Scutellarein	C15 H10 O6	-0.16	286.0477	285.0404	24.488	85.9	[M-H]-1	27092104.56	57137458.67
Quercetin 3-O-β-D-Glucuronide	C21 H18 O13	0.54	478.075	479.0824	21.015	73.2	[M+H]+1	27047028.43	16055904.91
Quercetin	C15 H10 O7	0.18	302.0427	301.0354	23.648	72.3	[M-H]-1	26903655.53	
Benzoic acid	C7 H6 O2	0.31	122.0368	123.0441	30.569	82.2	[M+H]+1	26165377.84	23592874.67
4-Hydroxybenzoic acid	C7 H6 O3	-0.8	138.0316	137.0243	22.84	79.2	[M-H]-1	24315609.68	
Alpinetin	C16 H14 O4	-0.31	270.0891	271.0964	27.993	74.1	[M+H]+1	22254196.62	30471970.9
Artemetin	C20 H20 O8	-0.16	388.1158	389.123	35.298	74.2	[M+H]+1	21879568.67	4923860.283
α-Boswellic acid α-	C30 H48 O3	0.12	456.3604	457.3678	31.06	73.6	[M+H]+1	21269491.59	5753882.64
Scopoletin	C10 H8 O4	0	192.0423	193.0495	21.946	77.2	[M+H]+1	20119662.39	40673439.02

Rutaevin	C26 H30 O9	-1.4	486.1883	487.1956	28.615	76.7	[M+H]+1	19705260.6	23766931.62
Vicenin III	C26 H28 O14	-0.06	564.1479	563.1405	20.018	86	[M-H]-1	19208344.62	23504794.6
Pinosylvin	C14 H12 O2	-0.33	212.0837	213.091	25.595	91	[M+H]+1	18611822.09	23505540.68
5-Hydroxy-6,7-dimethoxylflavone	C17 H14 O5	0.45	298.0843	299.0916	27.118	70.3	[M+H]+1	15433097.65	22430875.6
Glycitin	C22 H22 O10	0.12	446.1214	445.1141	23.605	72.1	[M-H]-1	14216084.1	6224365.958
Curcumol	C15 H24 O2	-0.33	236.1776	237.1848	27.312	78.8	[M+H]+1	14031614.77	31922531.89
Rutaecarpine	C18 H13 N3 O	-0.28	287.1058	288.1131	34.492	76.7	[M+H]+1	12951521.75	9483861.964
5-O-Demethylnobiletin	C20 H20 O8	-0.08	388.1158	389.1231	29.359	74.9	[M+H]+1	12923763.3	29359440.16
Isoacteoside	C29 H36 O15	0.26	624.2056	623.1983	22.167	80.3	[M-H]-1	12280366.2	
Mulberrin	C25 H26 O6	0.09	422.173	423.1801	41.282	72.4	[M+H]+1	11741939.99	19936834.05
Glabridin	C20 H20 O4	-0.35	324.136	325.1433	36.463	71	[M+H]+1	2343825.143	4773490.293
Complanatuside	C28 H32 O16	0.12	624.1691	623.1621	22.537	72.3	[M-H]-1		19265628.05
Oroxin A	C21 H20 O10	-0.16	432.1056	431.0983	24.726	77	[M-H]-1		11800922.65
1-Caffeoylquinic acid 1-	C16 H18 O9	-0.28	354.095	353.0877	17.032	83.3	[M-H]-1		10605841.5
Irigenin	C18 H16 O8	0.08	360.0846	359.0773	28.977	73.2	[M-H]-1		24080693.47
Oxysophocarpine	C15 H22 N2 O2	0.16	262.1682	263.1755	17.05	77.1	[M+H]+1		20800299.93
Pinoresinol 4-O-glucoside (+)-	C26 H32 O11	0.16	520.1946	565.1926	22.309	77.9	[M+FA-H]-1		55356017.39
Betaine 甜菜碱	C5 H11 N O2	0.02	117.079	118.0863	63.517	78.2	[M+H]+1		98924693.8
α-Cyperone α-	C15 H22 O	0.07	218.1671	219.1744	29.098	82.4	[M+H]+1		23793115.49
Eupafolin	C16 H12 O7	0.09	316.0583	317.0657	25.126	73	[M+H]+1		20430771.91
Tectorigenin	C16 H12 O6	-1.28	300.063	301.0703	28.569	74.7	[M+H]+1		624186748.4
L-Phenylalanine L-	C9 H11 N O2	0.39	165.079	166.0863	10.692	84.3	[M+H]+1		1201258069
Prim-O-glucosylcimifugin	C22 H28 O11	0.33	468.1633	469.1706	20.56	79.5	[M+H]+1		102820207.3
Isorhamnetin	C16 H12 O7	0.11	316.0583	315.0511	26.439	79	[M-H]-1		19222480.64
5,7-Dihydroxychromone 5,7-	C9 H6 O4	-0.42	178.0265	177.0193	22.833	72.1	[M-H]-1		15981715.79
Dihydrosanguinarine	C20 H15 N O4	-0.44	333.1	334.1072	25.376	72.3	[M+H]+1		959789688.9
Chrysophanol 8-O-β-D-glucoside	C21 H20 O9	0.51	416.1109	415.1037	24.832	79.5	[M-H]-1		21387110.14
Emodin-3-methyl ether/Physcion	C16 H12 O5	-1.09	284.0682	285.0754	23.531	77.6	[M+H]+1		29119543.28
Baicalin methyl ester	C22 H20 O11	-0.28	460.1004	461.1077	24.894	84	[M+H]+1		87901531.48
Cimifugin	C16 H18 O6	-0.84	306.1101	307.1176	22.011	86.5	[M+H]+1		165322440.8

Table 2: Blood routine test results of male rats in each group (n=10)

Items	control	High dose	Medium dose	Low dose
WBC(10 ^{9}/L)	6.22±1.41	6.64±1.12	7.04±1.08	6.62±1.14
RBC (10 ^{9}/L)	6.98±0.58	6.89±0.53	7.20±0.64	7.09±0.50
HGB (g/L)	144.7±5.03	151.80± 7.67*	145.2±5.07	144.4±4.50
HCT (%)	40.95±1.37	42.51±1.92	40.84±1.88	40.74±1.40
MCV (fL)	58.95±4.41	62.17±6.78	57.19±6.61	57.69±3.93
MCH (pg)	20.81±1.32	22.19±2.29	20.33±2.28	20.46±1.57
MCHC (g/L)	353.4±7.05	357.2±12.85	356.2±19.75	354.7± 12.01
RDW (%)	12.61±0.65	12.63±0.43	12.32±0.52	12.33±0.55
PLT (10 ^{9}/L)	922.4± 46.61	912.4±43.1	912.6±55.43	933.5± 40.95
PCT (%)	53.78±3.27	51.66±2.69	52.58±5.04	56.56±5.31
MPV (fL)	5.84±0.40	5.68±0.42	5.76±0.40	6.06±0.51*
PDW (%)	16.38±0.41	16.54±0.36	16.46±0.39	16.61±0.40
LYM (10 ^{9}/L)	4.98±0.81	5.37±0.98	5.50±0.72	5.17±1.05
MON(10 ^{9}/L)	0.14±0.81	0.17±0.08	0.15±0.09	0.16±0.07
Gran (10 ^{9}/L)	1.45±0.27	1.43±0.30	1.56±0.31	1.56±0.31
LYM (%)	80.97±6.50	80.90±6.98	78.51±5.59	77.79±3.45
MON (%)	2.36±1.00	2.51±1.02	2.19±1.28	2.43±0.99
Gran (%)	23.66±3.35	21.73±4.23	22.16±2.57	23.54±1.93

The values are presented as means±standard errors of the mean (n = 10).
* Significance vs. the control group: P<0.05.

Determination of maximum dosage: The cumulative dosage administered to the mice within 1 day was 128g/kg. After the administration of the herbal medicine, the mice exhibited symptoms of lethargy due to stress. However, they resumed normal eating and activity within 4 hours. Over the course of 14 days, no deaths or pathological phenomena were observed in the mice. The variations in body weight of the experimental group were not statistically significant when compared to the control group (P>0.05) (Fig. 2). Upon dissection, no visible pathological changes were observed in the major organs. The maximum dosage of this herbal compound was determined to be 128g/kg.

Subacute oral toxicity

General observation and mortality: Daily oral administration of JWSHT at concentrations of 4, 8 and 16g/kg/day showed no significant behavioral changes in the rats compared to the control group. The rats were alert, with even breathing patterns. On the second day after dosing, three rats from the middle dose group exhibited clustering behavior, ruffled fur, and lethargy. Additionally, one rat from this group showed symptoms of soft stool and other discomforts. However, by the third day, these symptoms had subsided and returned to normal. Throughout the experimental period, no rats died, resulting in a mortality rate of 0%.

Body weight and feed intake: After 30 days of oral administration in rats, there was no significant difference in body weight between the three herbal formula dose groups and the control group (P>0.05) (Fig. 2). There was no difference between males and females, indicating that the herbal formula had no significant impact on the growth and development of the rats.

Hematological parameters: Compared to the control group, the high-dose group showed an increase in hemoglobin (P<0.05), and the low-dose group exhibited a rise in mean platelet volume (P<0.05). However, there were no significant differences in other indicators between the various dose groups and the control group (P>0.05). (Table 2, 3).

Biochemical parameters: Compared to the control group, the alanine aminotransferase (ALT) levels in the low-dose group of rats decreased (P<0.05). The differences in the CREA, BUN, and AST indicators between each dosage group and the control group were not statistically significant. (P>0.05). (Table 4, 5).

Organ-to-body weight ratio: The results showed that after gavage, the relative spleen weight of the male high-dose group was significantly higher than that of the control group (P>0.05) (Fig. 3). There were no significant

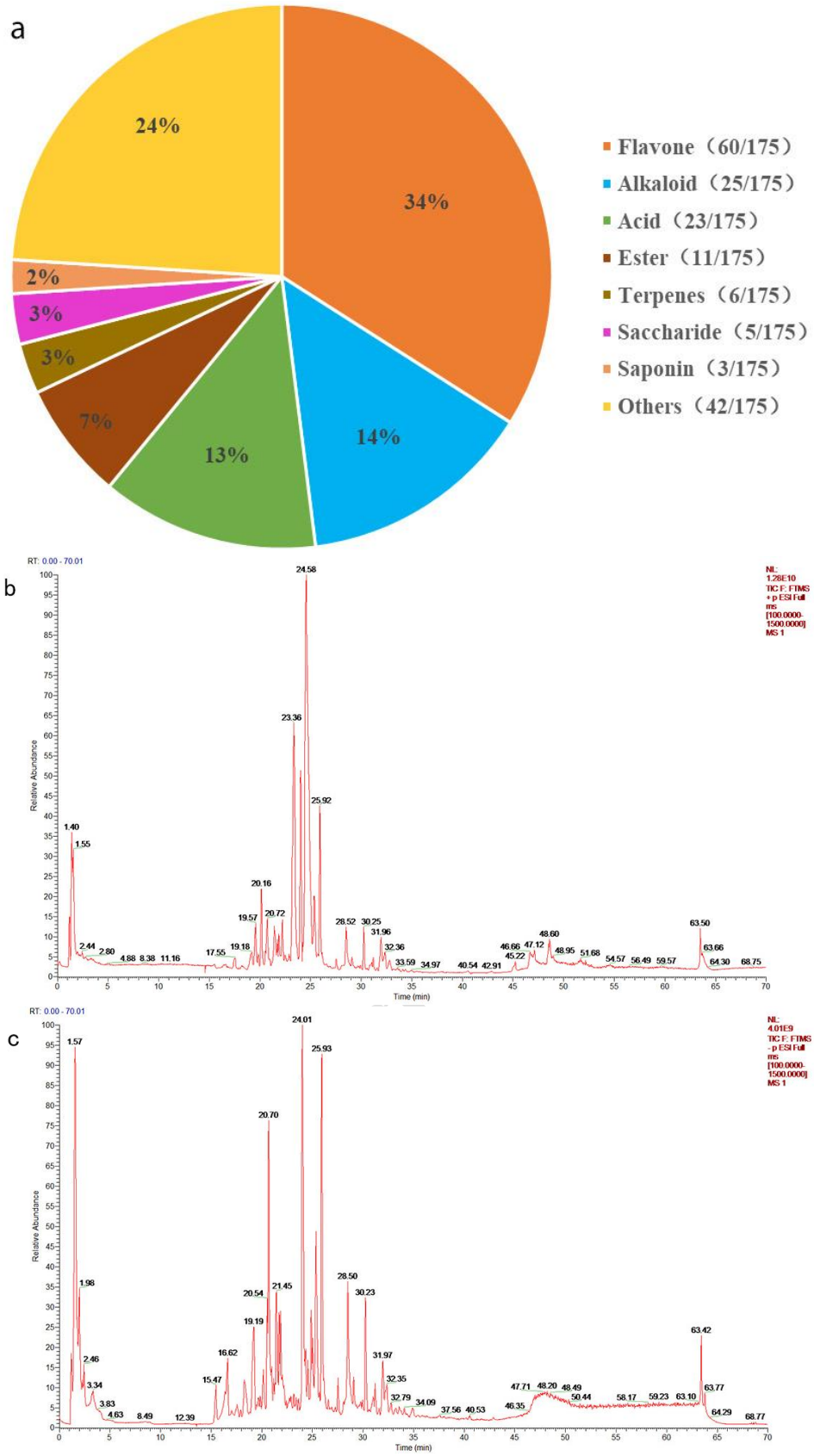


Fig. 1: The proportion of components(a) of JWSHT and TIC chromatograms under positive(b) and negative(c) ions modes.

differences in the relative weight of other organs (liver, heart, lungs, kidneys, stomach, and duodenum) compared to the control group.

Histopathologic analyses of vital organs: After gavage, the rats were dissected, and the size and color of the main organs were similar to those of the control group, with no visible hemorrhagic spots, swelling, necrosis, or other gross pathological changes. Under the optical microscope, histopathological sections of rat tissues showed a small amount of red blood cell infiltration in the kidneys of individual rats in the high-dose group, but there were no significant differences in other organs compared to the control group. (Fig. 4).

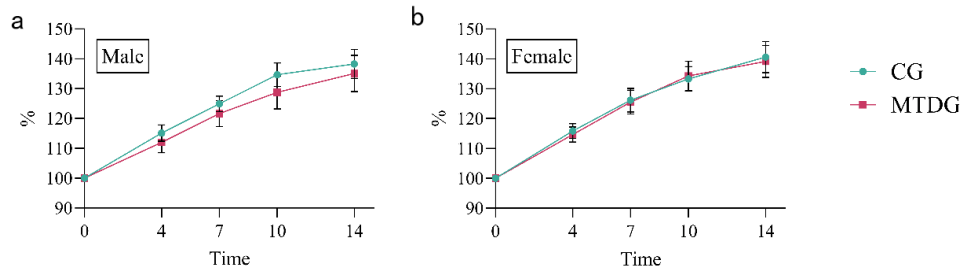
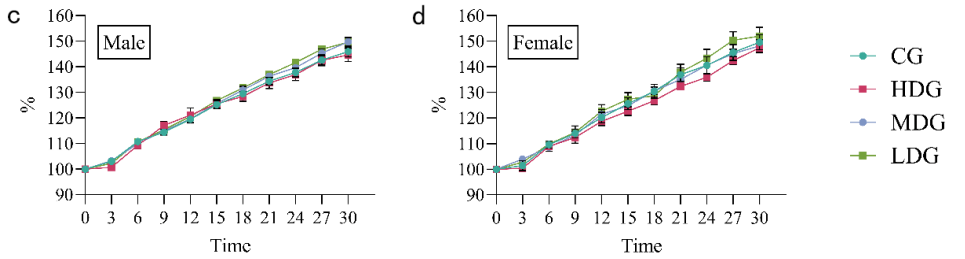


Fig. 2: Body weights of treatment groups that received acute (a, male; b, female) or subacute (c, male; D, female) treatment with JWSHT.



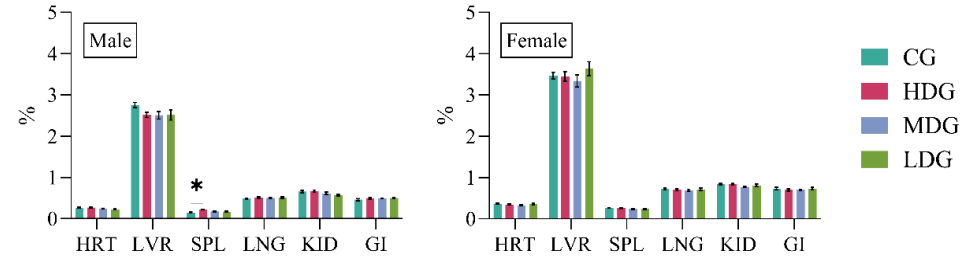


Fig. 3: Effect of Chinese herbal compound on organ-to-body weight ratio in rats(n=10), * Significance vs. the control group: P < 0.05.

Table 3: Blood routine test results of female rats in each group (n=10)

Items	control	High dose	Medium dose	Low dose
WBC (10^9/L)	6.64±1.34	6.87±1.17	6.29±1.40	6.60±0.91
RBC (10^9/L)	7.23±0.32	7.18±0.62	6.71±0.48	7.12±0.45
HGB (g/L)	144.2±4.98	150.00± 7.08*	144.5±4.28	145.7±3.43
HCT (%)	40.84±1.41	41.99±2.02	41.33±1.04	41.07±0.96
MCV (fL)	56.54±2.93	58.82±5.26	61.77±3.56	57.85±3.77
MCH (pg)	19.96±0.96	21.01±1.78	21.59±1.22	20.53±1.36
MCHC (g/L)	353.2± 10.52	357.5±15.29	349.6±5.24	354.9±9.08
RDW (%)	12.48±0.57	12.58±0.33	12.51±0.43	12.37±0.54
PLT (10^9/L)	917.7± 59.76	920.1±44.7	909.7±45.82	910.5± 39.78
PCT (%)	51.85±3.66	52.96±4.16	51.82±3.79	56.59±4.31
MPV (fL)	5.66±0.38	5.76±0.41	5.70±0.36	6.23±0.50*
PDW (%)	16.46±0.49	16.46±0.49	16.81±0.41	16.46±0.43
LYM (10^9/L)	5.26±1.25	5.51±0.85	5.70±0.36	5.25±0.98
MON(10^9/L)	0.17±0.08	018±0.08	0.16±0.07	0.16±0.05
Gran (10^9/L)	1.51±0.36	1.64±0.37	1.46±0.30	1.46±0.16
LYM (%)	78.96±5.79	80.63±7.01	80.31±6.99	79.16±5.04
MON (%)	2.60±1.24	2.59±1.05	2.65±1.19	2.52±0.99
Gran (%)	22.75±2.64	23.71±2.45	23.52±3.29	22.29±2.42

The values are presented as means±standard errors of the mean (n = 10).
* Significance vs. the control group: P < 0.05.

Table 4: Blood biochemical test results of male rats in each group(n=10)

Items	Control	High dose	Medium dose	Low dose
CREA	75.21±9.37	75.02±8.52	74.56±9.36	73.49±9.46
BUN	6.72±0.57	6.53±0.54	6.31±0.53	6.47±0.69
ALT	48.42±2.62	48.26±3.05	47.89±2.92	42.91±3.78*
AST	176.8±8.69	175.9±8.80	179.0±10.51	176.3±10.55

The values are presented as means±standard errors of the mean (n = 10).
* Significance vs. the control group: P < 0.05.

Table 5: Blood biochemical test results of female rats in each group(n=10)

Items	Control	High dose	Medium dose	Low dose
CREA	73.48±7.99	74.37±8.37	72.28±7.03	72.29±6.88
BUN	6.63±0.60	6.74±0.53	6.38±0.74	6.58±0.48
ALT	47.66±2.34	48.06±1.92	46.70±3.32	42.31±3.53*
AST	175.4±13.31	172.8±11.25	177.7±10.95	178.4±9.73

The values are presented as means±standard errors of the mean (n = 10).
* Significance vs. the control group: P < 0.05.

DISCUSSION

In recent years, medicinal plants have garnered significant attention due to their pharmacological effects. However, the toxicity of their active ingredients remains not fully elucidated. Their potential toxicity has become a serious medical concern. Therefore, this study aims to assess the acute and sub-acute toxicity of JWSHT, providing guidance for its safe clinical application.

The chemical complexity of JWSHT not only reveals its rich pharmacological effects but also provides crucial information for our assessment of acute and sub-acute toxicity. Through HPLC-MC technology, we identified that this formulation primarily contains 175 chemical components, especially flavonoids, alkaloids, and acids, which are well-known for their extensive pharmacological activities. The results show that flavonoids account for 34% of the total, alkaloids for 14%, and acids for 13%. Commonly found in plants, flavonoids have been proven to have antioxidant, anti-inflammatory, and anti-tumor effects (Imran *et al.*, 2019; Singh *et al.*, 2020; Chagas *et al.*, 2022). Their antioxidant potential can mitigate cell damage caused by free radicals (Gupta *et al.*, 2022; Carlini *et al.*, 2022), while alkaloids exhibit analgesic and anti-inflammatory properties (Gao *et al.*, 2022; Zhang *et al.*, 2022), contributing to the overall safety of the drug. Therefore, the characteristics of these components are crucial for assessing the safety of JWSHT, and it is necessary to establish an HPLC-MC method to determine the structure and chemical properties of JWSHT.

In the acute study, we tested four dosage groups. The highest dosage (1.6g/kg) was the maximum concentration tolerated by mice. At the end of the experiment, no significant organ abnormalities were found during the

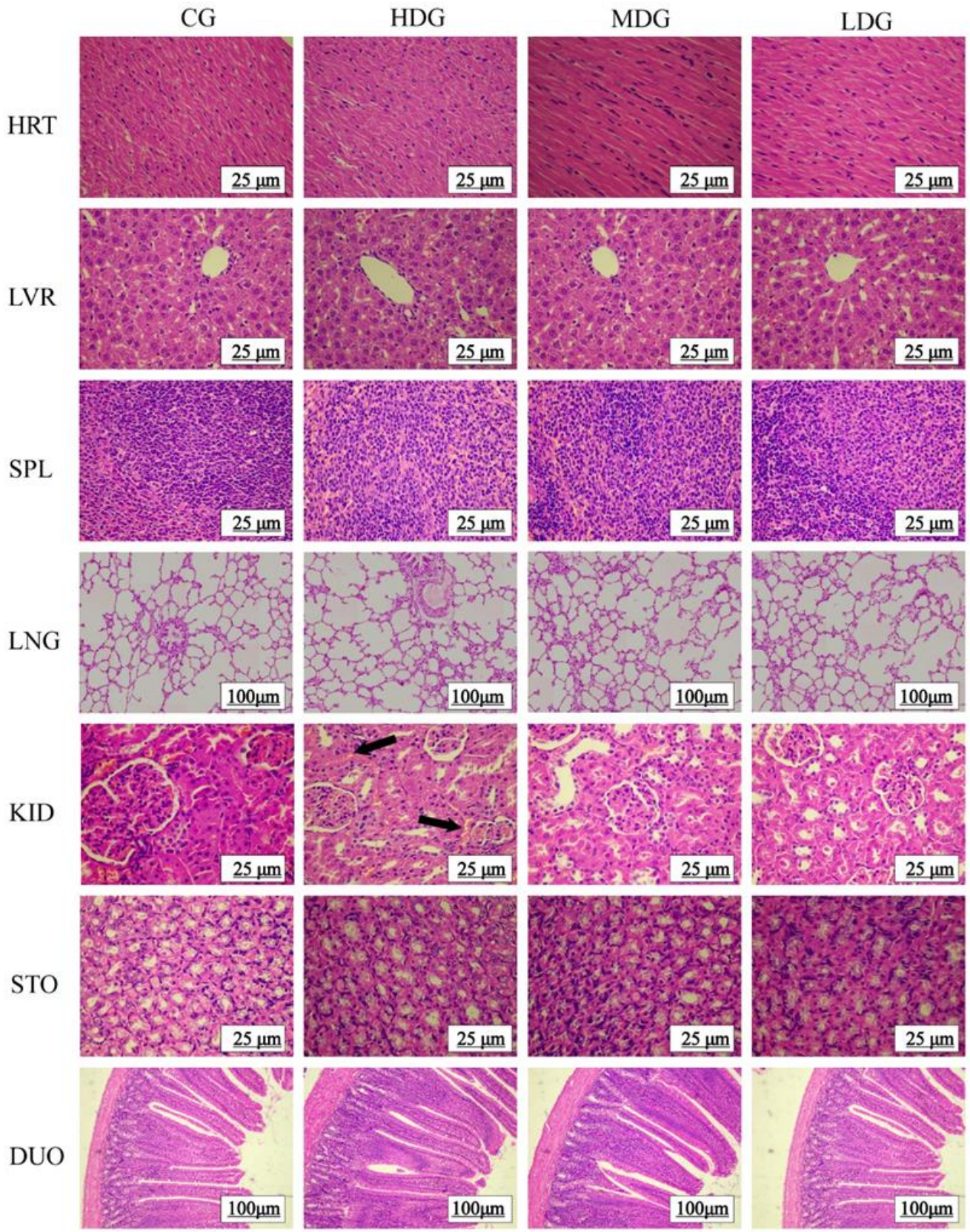


Fig. 4: Tissue sections of main organs of rats in each group. The arrows indicate a slight infiltration of red blood cells.

autopsy, and there were no obvious toxic reactions in mice. Therefore, it can be inferred that the LD50 of JWSHT in mice is much higher than 1.6g/kg. Further tests conducted at higher cumulative dosages also did not reveal any significant physiological or behavioral abnormalities. According to the OECD standards, with an LD50 > 5g/kg, JWSHT can be classified as essentially non-toxic (OECD, 2002).

After completing the acute toxicity test, long-term toxicity tests are necessary. According to the dosage guidelines in the 'Methodology of Pharmacological Research on Traditional Chinese Medicine', the high-dose

group is designed based on clinical dosages to determine the safe dosage range for animals. Therefore, for this sub-acute toxicity test, 4g/kg was chosen as the low dosage group, and dosages were increased proportionally, divided into three dosage groups. After 30 days of continuous administration, there were no deaths in any of the treatment groups among the rats.

Body weight is considered a sensitive indicator of drug toxicity (Deyno *et al.*, 2020; Wu *et al.*, 2022; Canh *et al.*, 2023), and no abnormal changes were observed in this study. Relative organ weight, especially the relative spleen weight, is considered an important indicator reflecting the immune function status of animals (Kang *et al.*, 2021; Yan *et al.*, 2021). The results showed that the relative spleen weight of the high-dose group was significantly higher than that of the control group, but no histopathological abnormalities were found. Astragalus polysaccharides, as the main component of *Astragalus*, can stimulate macrophage activity and increase the secretion of immune cell cytokines, thereby enhancing immune function (Li *et al.*, 2022); Berberine, as the main component of *Coptis*, can affect the activation and secretion of lymphocytes, thereby regulating the immune system (Ehteshamfar *et al.*, 2020). This suggests that JWSHT may cause an increase in spleen weight through immunomodulatory effects, indicative of enhanced immune response rather than pathological changes.

Additionally, from the hematological parameters, compared with the control group, the MPV value of the high-dose group significantly increased, suggesting enhanced activation and aggregation ability of platelets, and the HGB value of the high-dose group also showed an increasing trend, indicating an improvement in the quantity or quality of red blood cells. This could possibly be influenced by flavonoids and polysaccharides, as these substances can regulate platelet function by improving microcirculation or affecting components of the blood coagulation system (Zaragozá *et al.*, 2021; Zaragozá *et al.*, 2022; Araujo *et al.*, 2023). These preliminary findings suggest that JWSHT may have an impact on the blood system, but the specific mechanisms and long-term effects require further study. Particularly, its impact on platelet function may need to be explored through more detailed experiments.

As a liver function marker, the decrease in ALT at a dosage of 4g/kg suggests that JWSHT might reduce liver metabolic capacity. However, no significant abnormalities such as hepatocellular degeneration or steatosis were observed in the histological examination of the liver (Xu *et al.*, 2020; Choaib *et al.*, 2023). Flavonoids and polyphenols have strong antioxidant effects, capable of mitigating cell damage caused by free radicals, thus protecting the liver (El-Aarag *et al.*, 2019; Zhao *et al.*, 2021). This suggests that JWSHT may have a potential protective effect on the liver, leading to reduced liver cell damage and consequently a decrease in ALT (Xu *et al.*, 2018). However, given the observation of red blood cell infiltration in the kidneys of the high-dose group, we cannot entirely rule out the potential toxic risks of long-term administration or higher doses of JWSHT.

It is important to note that the components and mechanisms of action of traditional Chinese medicine compound formulations are very complex. There may be interactions between different components, and the ways in which they affect the body can vary greatly. This study provides preliminary evidence for the safety of JWSHT. Therefore, to ensure its safety in clinical applications, these preliminary results need to be validated and further explored through more extensive research.

Conclusions: The results of the acute test indicate that the LD50 of JWSHT is greater than 5g/kg, classifying it as essentially non-toxic. The sub-acute test results show no significant pathological changes after 30 days of administration at various doses. The study suggests that JWSHT is safe for clinical use.

Authors' contributions: WLY and CZ conceived the research idea, and both individuals made equal contributions. Professor WJQ developed the concept, monitored and mentored the proposal development. Professor YG. has polished this article. WLY、CZ、DZM and LZQ conducted the experiment. All authors have read and agreed to the published version of the manuscript.

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