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Study on Antiviral *in Vitro* And Long-Term Toxicity to Rats of the Fruit from Prunus *Cerasifera Ehrhar F*

Chen Xiao Shi, Hao Guo, Yan Xue Zhang and Chang Zheng Zhou*

Department of Medicine, Shandong University of Traditional Chinese Medicine, China

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***Corresponding author:** Chang Zheng Zhou, Department of Medicine, Shandong University of Traditional Chinese Medicine, Jinan, China

Abstract

Background: In recent years, many literatures have pointed out that the fruit of Prunus *cerasifera Ehrhar f.* has anti-oxidation effect. Based on the anti-oxidation principle of traditional Chinese medicine in antiviral aspect the antiviral effect of the fruit was studied herein and the problem of the toxicity of the fruit has not been systematically investigated yet. To clarify the toxic effects of the fruit and its material basis, the toxicity test of the fruit was carried out.

Methods: The fruit was percolated with water and the percolated liquid was concentrated to collect the concentrated liquid. Viral infection model *in vitro* was used to evaluate the antiviral effects of different viruses *in vitro.* CPE was observed under a microscope to determine the inhibitory effects of different viruses. The best type of macro-porous resin was determined by the method of macro-porous resin adsorption. The types of active ingredients were determined by physical and chemical identification, and their contents were determined by UV. By intragastric administration the long-term toxicity of percolating juice of the fruit to rats was observed including the nature severity, dose-toxicity relationship, time-toxicity relationship, main target organs, toxicity reversible degree, delayed toxicity, etc.

Results: showed that percolation solution of the fruit had obvious inhibitory effect on HSV-1 virus, and the TI value was 109.22. The inhibition effect of 60% ethanol precipitation on HSV-1 was the best, and the TI value was 148.84. D101 macro-porous resin presented an excellent separation effect at the volume of the first column eluted by 25% ethanol and TI value was 200.85. Chemical polyphenols were determined by physical and chemical identification and the content was 18.15 mg/g. Comparison of each dose group of percolation of the fruit and blank group there were not abnormalities in appearance signs, behavioral activities, body weight, food intake, hematological examination, serum biochemistry, organ quality and organ coefficient; Morphological examination of organs such as heart, liver, spleen, lung, kidney, ovary, testis, etc. didn't show pathological changes related to medication; Reversible observation showed that the fruit did not cause delayed toxicity to rats. There was not statistically significant difference in the above indicators.

Conclusion: Polyphenols from 25% alcohol elution site of percolation of the fruit may have good anti-HSV-1 activity. The large dose of percolation of the fruit and long-term use don't have obvious toxicity which provides a basis for clinical application.

Keywords: Prunus *cerasifera Ehrhar f.*; Antiviral; Long term toxicity; Safety

Introduction

Prunus *cerasifera Ehrhar f.*, also known as red leaf plum, is a medicinal and edible plant of the genus Prunus in the rose family. Its fruits are small and astringent, which are mostly scattered naturally after ripening and rarely used for fresh food [1]. Purple leaf plum as an ornamental plant, luxuriant branches and leaves, is widely planted in parks gardens and other places has great ornamental value. However, the fruits of purple plum are mostly scattered directly on the ground, and their application value and prospect are greatly underestimated. Purple leaf plum fruit as a natural antioxidant has medicinal and health care value (protecting liver nourishing the middle and benefiting qi, nourishing Yin and promoting body fluid moistening intestines and laxative, etc. [2]). Its main chemical constituents include total flavonoids [3-5], total polyphenols [6-8] and some other chemical constituents [9], such as beta-carotene (4) and quercetin. It has strong anti oxidant capacity [10-11] and protective effect on alcoholic liver injury (ALD) rats [12]. Despite its many benefits, its antiviral and long-term toxicity have not been studied. TCM antiviral drugs are widely used in the Asia-pacific region, especially in China. At present, the separation of chemical components of traditional Chinese medicine is basically systematic separation method. After various means and technologies are processed, some specific substances are obtained, and then these substances are screened by later activity tests. However, the specific substances obtained do not necessarily have pharmacological activity which wastes a lot of manpower, financial resources and material resources. In this study we used the method of *in vitro* antiviral activity tracking to conduct antiviral tests on the isolated and purified substances in each step of the test and took the therapeutic index as the direction to verify whether the site obtained in this step has the antiviral ac-

tivity we want. This kind of targeted and targeted separation of active parts of traditional Chinese medicine is obviously superior to the traditional method of separating certain substances.

The mechanism of antiviral action of traditional Chinese medicine can be summarized as: killing virus directly or reducing virus virulence; To prevent the absorption and invasion of viruses; Inhibits the transcription and replication of viral DNA or m RNA; Oxidation resistance; Regulate the production of cytokines, etc. Based on the antioxidant effect of the fruit and the anti-oxidative principle of traditional Chinese medicine in antiviral aspect, the antiviral effect of the fruit was preliminarily studied. At the same time why, few people eat the fruit is because of its astringency or some toxicity then the toxicity test of the fruit was carried out. To observe the long-term toxic effect of percolation of the fruit on rats the test was carried out. It includes the nature of reaction, severity, dose-toxicity relationship, time-toxicity relationship, main target organs, toxicity reversibility, delayed toxicity, etc. It provides reference for its edible and clinical use.

Test Materials

Test equipment

carbon dioxide cell incubator was bought from FOMAS; carbon dioxide virus incubator was bought from Shanghai Yue Jin medical equipment co., LTD.; clean bench was bought from Shanghai Li Shen scientific instrument co., LTD.; High speed desktop centrifuge was bought from TD5M, Changsha Xiang Zhi centrifuge instrument co., LTD.; Fluorescence inverted microscope and Ckx-31 inverted microscope was bought from Olympus company; MK3 Eliasa was bought from Lab systems; 96-well cell culture plate was bought from Corning, USA; Liquid nitrogen tank was bought from Sichuan liquid nitrogen tank plant. YP1200 electronic balance; French ABX Pentra 60 hematocyte analyzer; Japan Olympus BX41 microscope; Japan Hisen Meikang CA1500 automatic blood coagulation instrument; Japan OLYMPUS AU640 automatic biochemical analyzer; Easy Lyte PLUS sodium/potassium/chlorine analyzer of MEDICA CORPORATION, USA; LD25-2 low-speed automatic balancing centrifuge.

Test reagents

D101macroporousresins, AB-8macroporous resins, DM-301macroporous resins and X-5macroporous resins was bought from Chemical plant of Nanjing university; 1640 Cell culture medium (GIBCO, 10% bovine serum); 1640 Cell maintenance solution (GIBCO, 2% bovine serum). phosphate buffer solution (PBS) of PH 7.4 (containing NaCl 8g, KC l0.2g, KH_2PO_4 0.2g, Na_2HPO_4 2.9g add distilled water to 1L,filtered and sterilized, placed in 4°C for later use); neutral red dye solution, neutral red decolorizing solution, gallic acid was bought from Shanghai Aladdin biochemical technology co., LTD.; phosphomolybdate was bought from Xiamen Hai Biao technology co., LTD.; other chemical reagents were analytically pure.

Efficient enzyme cleaner, enzymatic detergent II, blood hemolytic agent (acidophilus Lyse-ABX-EO) blood hemolytic agent (basophilic Lyse ABX-BA) blood hemolytic agent (hemoglobin Lyse-ABX-HGB) blood diluent the above reagents were provided by the Yantai Zhuoyue biological technology co., LTD. Blood glucose determination kit, triglyceride assay kit, total cholesterol assay kit, total bilirubin determination kit , total protein assay kit, albumin assay kit, alanine amino transferase assay kit, aspartate amino transferase Assay kit, alkaline phosphatase assay kit, urea assay kit, creatinine assay kit, creatine kinase assay kit, the above reagents were provided by Desay diagnostic system (Shanghai) co., LTD. Sodium/potassium/chlorine detection kits, MEDICA CORPORATION, United States.

Test cells and viruses

Cell lines and virus MA104 cells, RD cells (microbiology room, institute of basic medicine, Shandong academy of medical sciences); Respiratory Syncytial virus, enterovirus type 71, herpes simplex I viruses (Influenza virus laboratory, institute of virology, Chinese center for disease control and prevention)

Test animals

Wistar rats, SPF grade, 80, male and female half, provided by Jinan Peng Yue experimental animal breeding co., LTD., experimental animal production license no.: SCXK (Lu) 20140007. The rats were raised in rat cages, the number of each cage animal was 10, drinking water intake freely. Cage tools were exchanged twice a week. Feed room temperature was 22 ~ 26°C, humidity was 45~70%, and day and nighttime were 12h/12h, well ventilated. The feeding room was cleaned daily, and the bromo gramine was used to disinfect. Rats, feed was provided by Jinan Peng Yue experimental animal breeding co., LTD., with production license: SCXK (Lu) 2018-0003.And the rats were fed regularly and quantitatively. The drinking water and animal bedding were used after high pressure steam sterilization of 121°C, 30min.

Test drug

The fruit were picked in the campus of Shandong university of traditional Chinese medicine, Changqing district, Jinan city, Shandong province. The stone of the fruit were removed, the fruits were weighed for 100g and crushed. Percolated fruits with water as solvent, and then filtered it. The filtrate was concentrated to 1 g/mL (according to fruits' quality) and refrigerated for later use.

Test Method

Virus screening method

Cell resuscitation and culture: The cryopreserved tubes of MA104 cells and RD cells were picked up from the liquid nitrogen tank and then were putted in 37 ~ 40 °C warm water to melt with rapid stirring. (try to complete the dissolution within 1min). The cryopreserved tubes were centrifuged at 800r/min for 5min, and the cryopreserved liquid was removed on the ultra-clean Table 1. Added appropriate 10% 1640 cell culture solution to the cell precipitation and mixed well and transferred them to the culture bottle. Added 10% 1640 culture to the culture bottle to 12 ml, and then putted it at 37 °C and 5% CO_2 incubator. After the cells grew

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into a monolayer, the cells were digested with 0.25% trypsin, and the cells were passaged 1:2. When the cells grew into a monolayer the cells were used for the experiment [13].

Table 1:

The Index Name	Detection Method
RBC	Resistance method
HCT	Pulse method
HGB	Photoelectric colorimetry
MCV	Hematocrit /erythrocyte count×103
MCH	Hemoglobin/RBC count
MCHC	Haemoglobin/hematocrit
WBC	Resistance method
PLT	Resistance method
LYM%	Resistance method
NEU%	Resistance method
BAS%	Resistance method
EOS%	Resistance method
MON%	Resistance method
RC	The manual method
APTT	The solidification method

Virus amplification: RSV and HSV-1 virus were inoculated on the well-growing MA104 cells, and EV-71 enterovirus was inoculated on the well-growing RD cells. Added 2% 1640 cell culture solution to the cells and putted the cells at 37 ℃ and 5% CO_2 incubator for 24~48h.The cytopathic effect (CPE) was observed under the microscope and the experiment was stopped and collected when the CPE was 90%. Putted the culture bottle in the -20 ℃ environment with repeated freezing and thawing 3 times. Then the bottle was centrifugal (1000r/min, 5min), and quantitative packaging of supernatant fluid was putted into -40 ℃ environment for later use [14].

Determination method: The samples were diluted with 2% 1640 cell maintenance solution. A total of 12 dilutions were performed at 2 times serial dilutions. Three holes were set, and 50μL was added to each hole in the 96-well plate of monolayer virus host cells. 50μL RSV with 100-times $TCID_{50}$ was added to each well, and the virus control group, cell control group and drug toxicity group were set at the same time. Ribavirin was added to the positive control group and three wells were set. They were cultivated at 37 ℃ and 5% CO_2 incubator. Cell lesions were observed daily. When more than 90% lesions appeared in the viral control group, electron microscope was used to observe and find out the more effective sites. Patted the culture solution out of the 96-well plate. 100μL neutral red dye solution was added into every hole and then was putted in the condition of 37 ℃. They would be fetched out after 1h of reaction. When they were fetched out, neutral red dye solution was poured out. Next, they were rinsed 3 to 5 times with low flow. 100μL decolorizing solution was added to each well and was placed in the incubator for 15min. OD value was measured with an eliasa at 540nm. The antiviral experiments of EV-71 and HSV-1 *in vitro* were the same as above.

Determination of virulence of the virus: According to the conventional virulence measurement, the commonly used virus virulence measurement time of 48h was selected as the measurement time to determine the virulence required by the experiment. Reed-Muench formula was used to calculate the 50% infection concentration of the virus venom ($TCID_{50}$).

Determination method: The virus was diluted 12 times serial dilutions with the maintenance solution and the virus was successively inoculated in 96-well plates of monolayer host cells, and the cell control group was set at the same time. Then the virus was placed in 37 ℃ and 5% CO_2 virus incubator to develop and were observed day by day under an inverted microscope for 4 days. Next, they were dyed for 1h under the condition of 37 ℃ after added into 50μL 1% neutral red. At last, we discarded the dye solution washed the excess dye solution thoroughly with distilled water added 100μL decolorizing solution decolorized at room temperature for 10min, and measured OD value with an Elisa at 540nm wavelength [15].

Effect of percolation of the fruit on virus inhibition: Reed-Muench method was used to calculate the drug half-effective concentration (EC_{50}) and treatment index (TI).

$$EC_{50} = [Anti\ \log(\log p1 - pd)] \times \text{int}\,ial\ concentration \quad (1)$$

$$TC_{50} = [Anti\ \log(\log p2 - pd)] \times \text{int}\,ial\ concentration \quad (2)$$

$$TI = \frac{TC_{50}}{EC_{50}}$$

Precipitation of the fruit by different concentration of ethanol: Different volume fraction of ethanol was used to separate and purify the percolation of the fruit. The supernatant and the precipitated part were respectively used for the anti-virus experiment *in vitro*, and the absorbance value was determined by an Eliasa and the TI value was calculated.

Separation and purification of percolate by macro-porous resin

Pretreatment with macro-porous resin: According to the specific components in the fruit(flavonoids, polyphenols, procyanidins, etc.), non-polar or weakly polar resins were selected. D101 (non-polar), DM301 (weak polar), X-5 (non-polar) and AB-8 (weak polar) resins were weighed 200g respectively and screened to conduct antiviral tests. At first, they were soaked in 95% ethanol for 24h and the ethanol liquid level was 5cm higher than the resin. Then, they were packed into the chromatographic column by wet packing and washed with 95% ethanol. Then, we needed to check the outgoing liquid at any time and mix the liquid with water at 1:5. If the mixed solution was not white turbidity but clear and transparent, ethanol elution should be stopped. Finally, we rinsed the resin column with distilled water until the effluent didn't has alcohol taste. Set them aside.

The loading and elution of macro-porous resin: The ethanol precipitation with good antiviral effect under "2.2" was made into 20ml aqueous solution and added to the prepared macro-porous resin column. The aqueous solution of the drug was

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5cm higher than the resin and they were standing for 1h. After adsorption distilled water 25%, 50% and 75% ethanol were used for eluting. The volume flow was controlled to be 3 BV/h and each eluent received 5 column volumes. The eluent was concentrated to 5ml (equivalent to 1g/ml) respectively, for antiviral experiments *in vitro*.

Antiviral experiments: The ethanol precipitation was divided into four parts, and then dissolved in distilled water (10ml per part). Then they were respectively loaded onD101, DM301, X-5 and AB-8 resins. Next, they were eluted with distilled water 25%, 50% and 75% ethanol and the eluent were collected for anti-HSV-1 experiments [16].

Physicochemical identification: Physicochemical identification was performed on the volume eluent of the first column eluted by 25% ethanol of D101 macro-porous resin. Because the water solubility of the extracts was relatively large, only the physicochemical identification of tannins, brass, glycosides and alkaloids was conducted in the experiment, and the types of the chemicals were preliminarily determined.

Determination of total polyphenols: According to the modified determination method of tannin content in (Chinese Pharmacopoeia) the content of polyphenols was measured.

Long term toxicity test method [17-19]

Animal groups: Rats were fed adaptively and were observed for 5 days and then tested. General conditions of rats during adaptive feeding were observed daily. The rats were weighed on the 1st day and the last day during adaptive feeding period, and the results of weighing were recorded. The male and female rats were randomly divided according to body weight by hierarchical grouping. It was divided into 4 groups: blank control group, high dose group, medium dose group and low dose group of percolation of purple leaf plum fruit. Twenty rats in each group, half male and half female.

Route and method of drug delivery: In this study oral gavage was used for drug delivery, with high, medium and low doses. The maximum gavage of rats is 5ml, so the high-dose group was given 5ml or about 5g. The medium dose group was given 3ml or about 3g. The low-dose group was given 1ml or about 1g. The animals in the control group were given 5ml distilled water by gavage. The duration of drug delivery of 4 weeks was designed. The drug was given once a day in the morning and continuously for 4 weeks.

The determination method and frequency of each index detection

General symptoms and death: During the experiment, general observation was made twice a day before and after drug delivery to observe the appearance behavior and activity gland secretion, respiration, diet, feces, etc. of rats. If abnormal reactions (toxic reactions) were found detailed observation and records were made. If death was found, autopsy was conducted in time and anatomy and histopathological examination were performed.

Weight determination: Determination times: the rats were weighed every Monday morning during the period of drug delivery and measured the weight before the autopsy. If there were dying rats, weighed them before the autopsy. Determination method: weighed before drug delivery during drug delivery period. Weighed on an empty stomach before dissection.

Determination of material consumption: Determination times: from the beginning to the end of the test, each cage was measured once a day.

Determination methods: rats were given adequate feed every week. The amount of food intake of each group of rats was weighed out every day. Calculation method:

$$\textit{The amount of food intake of each group of rats per day} = \textit{The given amount} - \textit{The residual amount}$$

Determination of hematology indexes: Detection times: the end of drug delivery (4 weeks) and the end of convalescence (2 weeks) were observed.

Example number: 10 rats in each group, half males and half females.

Blood collection methods: fasting for 12h, blood was taken from the abdominal aorta.

Methods: hematological indexes included: WBC, RBC, HGB, PLT, RC, APTT (Table 1).

Determination of serum biochemical indexes

Detection times: the end of drug delivery (4 weeks) and the end of convalescence (2 weeks) were observed.

Example number: 10 rats in each group, half males and half females. Blood collection methods: fasting for 12 h blood was taken from the abdominal aorta.

Methods: serum biochemical indexes included: GLU, TG, CHOL, TBIL, TP, ALB, ALT, AST, ALP, BUN, CRE, CK, Na^+, K^+, Cl^- (Table 2).

Table 2:

The Index Name	Detection Method
GLU	Hexokinase method
TG	GPO-PAP method
CHOL	Enzyme colorimetry
TBIL	2, 4-dichloroaniline diazo method
TP	The biuret method
ALB	Bromocresol green process
ALT	UV continuous monitoring method
AST	UV continuous monitoring method
ALP	Rate method
BUN	Glutamate dehydrogenase method
CRE	Enzymatic method
CK	IFCC continuous monitoring method
Na+	Ion selective electrode method (direct method)
K+	Ion selective electrode method (direct method)
Cl-	Ion selective electrode method (direct method)

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The Test Results

Antiviral results *in vitro* of the fruit

The result of virulence of the virus

$$Determination\ of\ formula = Cell\ Survival\ rate = \frac{The\ hole\ A490 - Control\ hole\ A490}{Normal\ control\ group\ A490 - Control\ hole\ A490} \quad (5)$$

$$Cytopathic = 1 - Cell\ Survival\ rate \quad (6)$$

$$Cytopathic\ rate = \frac{Test\ hole\ A490}{Normal\ control\ group\ A490} \quad (7)$$

$$TCID_{50} = Anti\ \log\ (\log C + pd \times \log Cm) \quad (8)$$

Where $TCID_{50}$ is shown in Table 3.

Table 3: TCID50 of each virus.

Virus Type	The Host Cell	$TCID_{50}$
RSV	MA104	$10^{-4.1}$
EV-71	RD	$10^{-4.2}$
HSV-1	MA104	$10^{-1.8}$

The result of the fruit on virus inhibition

The results are as shown in Table 4. It could be seen from Table 4 that the percolation of the fruit has the best antiviral effect on HSV-1 *in vitro*, and the TI is 109.22.

Table 4: Effect of percolation of the fruit on virus inhibition.

Virus Type	TC_{50}	EC_{50}	TI
RSV	$2^{-3.20}$	$2^{-9.30}$	68.59
EV-71	$2^{-1.50}$	$2^{-6.40}$	29.77
HSV-1	$2^{-2.50}$	$2^{-10.87}$	109.22

Table 5: Inhibition of HSV-1 by different concentration of ethanol precipitation of the fruit.

Type of Ethanol Precipitation	TC_{50}	EC_{50}	TI
50%ethanol precipitation	$2^{-1.65}$	$2^{-8.20}$	93.73
60%ethanol precipitation	$2^{-2.40}$	$2^{-10.92}$	148.84
70%ethanol precipitation	$2^{-2.30}$	$2^{-9.40}$	137.19
80%ethanol precipitation	$2^{-3.50}$	$2^{-10.2}$	103.97
90%ethanol precipitation	$2^{-4.20}$	$2^{-10.3}$	68.59

The result of Inhibition effect precipitation of the fruit by different concentration of ethanol on HSV-1: The supernatant didn't have effect, and the results of precipitation part were shown in Table 5. It could be seen from Table 5 that the TI value of the 60% ethanol precipitation part of the percolation of the fruit was 148.84, which showed that the 60% ethanol percolation part of the percolation of the fruit had a very significant inhibition on HSV-1. The purpose of alcohol precipitation is to take the essence and discard the dregs. When the concentration is low, impurities cannot be removed. When the concentration is high, most impurities can be removed. But some of the active ingredients will be coated, thus reducing the amount of active ingredients in the solution [20]. It can be seen from the results that the liquid with 60% alcohol precipitation concentration retains the active ingredients to the greatest extent on the basis of removing impurities [21]. Then the 60% ethanol percolation was separated and purified by macro-porous resin.

The result of Inhibition effect of different elution sites of different resins on HSV-1

The results were as shown in Table 6. (50%, 75% ethanol elution didn't have antiviral effect, so they were omitted). The results showed that the first column volume of 25% ethanol in D101 macro-porous resin had the best antiviral effect and the TI was 200.85. The adsorption capacity of D101 macro-porous is generally strong for organic compounds without polarity or weak polarity. It can be seen from the above results that the active components of anti-HSV-1 in the fruit should not be weakly polar chemicals [22].

The result of physicochemical identification

In the experiment, ferric chloride reaction (phenolic hydroxyl), lead acetate reaction (phenols) and vanillin concentrated sulfuric acid reaction (polyphenols) were positive; the phenomena of hydrochloride magnesium -powder reaction (to identify flavonoids) Molish test (to identify sugars) and iodine-potassium iodide reaction (to identify alkaloids) were not obvious. Through physicochemical identification, it could be concluded that the chemical composition of the 1st column volume eluent eluted by 25% ethanol of D101 macro-porous resin was mainly polyphenols. Polyphenols are called "the seventh nutrients", which is found in some common plant foods. The Japanese study showed that the polyphenolic compound EGCG may block the expansion of the disease virus in the body, which will benefit the development of a new generation of disease-fighting drugs [23].

Table 6: Effects of different elution parts of different resins on the HSV-1TI values.

The Resin Type	Water					25% alcohol				
	1-1	2-2	3-3	4-4	5-5	2-1	2-2	2-3	2-4	2-5
D101	125.03	113.66	117.58	123.86	137.19	200.85	127.11	127.11	14.93	6.96
AB-8	133.74	115.87	36.76	84.45	*	51.63	11.63	*	13.93	*
X-5	161.44	*	90.51	*	157.59	92.41	45.25	5.66	*	*
DM301	122.86	94.01	*	111.43	59.71	58.89	14.93	5.46	*	*

Note: * means invalid.

The content of polyphenols: In the range of 2.05-10.03μg/ml, Y=31.653X+0.0535 (R2=0.9992), the linear relationship between absorbance and polyphenol content was good, the average recovery rate was 98.50% (RSD=0.55%), and the total average polyphenol content was 18.15 mg/g.

The results of long-term toxicity to rats of the fruit

General symptoms and death: During the whole test period (4-weeks drug delivery and 2-weeks convalescence) the rats in the dosing groups were generally in good condition, and abnormal symptoms weren't observed in the appearance and signs, behav-

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ior, activity, respiration, feeding, fur, secretions, feces, etc. of the rats, and death wasn't observed. There was not significant difference compared with the blank control group.

Weight determination: During the experiment the rats in the control group and the purple leaf plum fruit dose groups showed good development and gradually increased quality, and there was not statistical significance between the dose groups and the blank group. After 2-weeks drug withdrawal and recovery there was not statistically significant difference between each dose group and the blank group as shown in Figure 1 & 2.

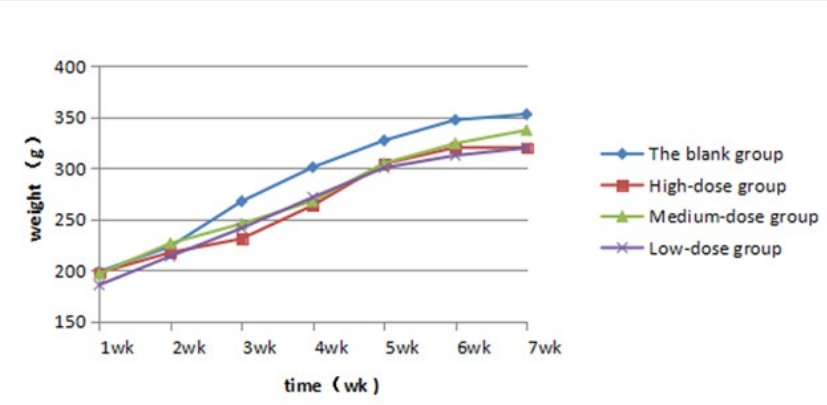

Figure 1: Effect of percolation of the fruit on weight of rats (male) during the period of 4-weeks drug delivery and 2-weeks convalescence (g/rat).

*Note: n=10 in each group before and after drug delivery of 1~4week and n=5 during the observation period of convalescence.

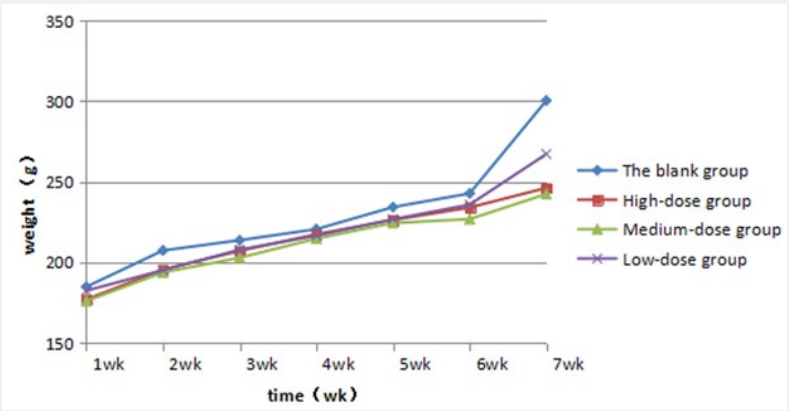

Figure 2: Effect of percolation of the fruit on weight of rats(female) during the period of 4-weeks drug delivery and 2-weeks convalescence (g/rat). *Note: n=10 in each group before and after drug delivery of 1~4week, and n=5 during the observation period of convalescence.

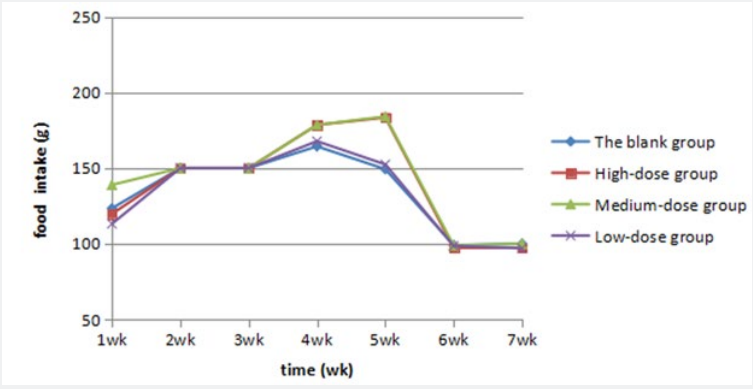

Figure 3: Effect of percolation of the fruit on intake of rats(male) during the period of 4-weeks drug delivery and 2-weeks convalescence (g/ day/group).

*Note: n=10 in each group before and after drug delivery of 1~4week and n=5 during the observation period of convalescence. Compared with the blank control group, P=0<0.05 at the 3rd week in the low-dose group. The rest of the P > 0.05.

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Determination of material consumption: In terms of dietary intake, there was not significant difference between the dose groups and the blank group at the drug delivery stage and the convalescence stage. As shown in Figure 3 & 4.

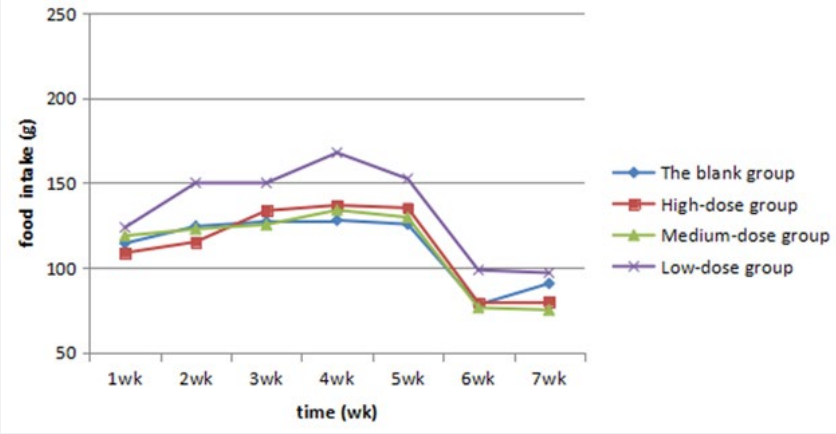

Figure 4: Effect of percolation of the fruit from on intake of rats (female) during the period of 4-weeks drug delivery and 2-weeks convalescence (g/day/group).

*Note: n=10 in each group before and after drug delivery of 1~4week and n=5 during the observation period of convalescence. Compared with the blank control group, P=0.04<0.05 at the 2nd week in the low-dose group. The rest of the P > 0.05.

Determination of hematology indexes: After 4-weeks drug delivery and 2-weeks convalescence, the hematological indexes of the rats in the low-dose, medium-dose and high-dose groups were all within the normal range with no statistical significance compared with the control group except for individual indicators of individual dose groups as shown in Table 7 & 8.

Table 7: Effect of percolation of the fruit on hematological indexes after 4-weeks drug delivery in rats

Groups	WBC	The WBC Classification(%)					RBC	HGB	HCT	MCV	MCH	MCHC	PLT	RC	APTT
	(109/L)	NE	LY	MO	EO	BA	(10^{12}/L)	(g/L)	(L/L)	(FL)	(pg)	(g/L)	(109/L)	(%)	(s)
The blank group	3.25± 2.32	8.04± 3.71	86.58± 2.92	4.62± 3.08	0.28± 0.29	0.48± 0.29	7.89± 0.5	146.6± 7.37	42.7± 2.56	54.34± 0.59	18.58± 0.33	343.4± 6.88	710.2± 43.44	2.48± 0.4	21.4± 0.4
Low -dose group	2.83± 2.28	10± 8.27	86.26± 8.18	2.84± 1.16	0.29± 0.36	0.61± 0.37	7.62± 0.43	141.25± 6.96	41.25± 2.05	53.03± 3.41	18.56± 0.49	342.5± 4.6	705.13± 230.79	4.01± 1.24	21.74± 0.9
Medium- dose group	3.12± 2.76	11.76± 6.36	84.31± 7.25	3.22± 1.81	025± 0.11	0.66± 0.43	8± 0.3	145.7± 5.83	42.76± 1.62	53.43± 0.9	18.22± 0.34	340.8± 3.85	790.6± 66.66	3.83± 1.08	21.85± 2.13
High-dose group	2.95± 2.37	7.94± 3.83	88.58± 4.87	2.76± 1.43	0.28± 0.14	0.65± 0.69	7.99± 0.41	147.75± 8.99	43.66± 2.76	54.6± 1.33	18.49± 0.44	338.5± 2.83	771.75± 101.23	3.51± 0.66	25.93± 9.67

*Note: n=10, half male and half female, compared with the blank control group: in high-dose group, MO: P=0.048<0.05; The rest of the P > 0.05.

Table 8: Effect of percolation of the fruit on hematological indexes after the period of convalescence in rats.

Groups	WBC	The WBC Classification(%)					RBC	HGB	HCT	MCV	MCH	MCHC	PLT	RC	APTT
	(10^9/L)	NE	LY	MO	EO	BA	(10^{12}/L)	(g/L)	(L/L)	(FL)	(pg)	(g/L)	(10^9/L)	(%)	(s)
The blank group	3.25± 2.32	8.04± 3.71	86.58± 2.92	4.62± 3.08	0.28± 0.29	0.48± 0.29	7.89± 0.5	146.6± 7.37	42.7± 2.56	54.34± 0.59	18.58± 0.33	343.4± 6.88	710.2± 43.44	2.48± 0.4	21.4± 0.4
Low -dose group	5.91± 1.76	6.49± 2.97	86.35± 3.46	6.48± 2.96	0.15± 0.07	0.35± 0.07	8.19± 0.59	147.75± 10.9	44.09± 3.81	53.54± 1.76	18.14± 0.34	337.25± 7.11	759.75± 81.84	23.06± 1.43	26.63± 4.17
Medium- dose group	5.1± 1.08	6.18± 2.46	86.2± 4.68	6.78± 2.78	0.12± 0.02	0.32± 0.01	7.74± 0.7	141.63± 10.38	41.84± 2.63	54.2± 1.62	18.34± 0.45	338.38± 4.96	779.88± 97.22	24.58± 1.65	24.23± 6.68
High-dose group	5.89± 3.07	5.9± 1.58	87.35± 2.6	6.31± 1.97	0.11± 0.02	0.42± 0.02	7.98± 0.15	146.13± 4.29	43.55± 1.08	54.58± 0.98	18.31± 0.3	332.55± 5.23	801.13± 80.2	25.53± 1.32	23.64± 3.64

*Note: n=10, half male and half female, compared with the blank control group: in high-dose group, EO: P=0.037<0.05; in low-dose group, WBC:P=0.043<0.05. The rest of the P > 0.05.

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Determination of serum biochemical indexes: After 4-weeks drug delivery and 2-weeks convalescence, the serum biochemical indexes of the rats in the low-dose medium-dose and high-dose groups were all within the normal range with no statistical significance compared with the control group, except for individual indicators of individual dose groups, as shown in Table 9 & 10.

Table 9: Effect of percolation of the fruit on serum biochemical indexes after 4-weeks drug delivery in rats.

Groups	GLU (m mol/L)	TG (m mol/L)	CHOL (m mol/L)	TBIL (u mol/L)	TP (g/L)	ALB (g/L)	ALT (U/L)	AST (U/L)	ALP (U/L)	BUN (m mol/L)	CRE (u mol/L)	CK (U/L)	K^+ (m mol/L)	Na^+ (m mol/L)	Cl^- (m mol/L)
The blank group	5.71± 1.97	0.65± 0.21	2.42± 0.97	12.1± 10.64	60.38± 8.18	36.03± 5.36	43.38± 11.21	173.13± 41.03	87± 49.26	7.75± 0.52	6.5± 18.42	1646.5± 1859.77	10.67± 2.71	131.75± 3.99	101± 2.88
Low -dose group	4.75± 1.32	0.58± 0.15	2.15± 0.62	5.34± 9.7	58.24± 3.8	33.99± 2.51	65.75± 42.72	270.63± 266.46	118.5± 33.01	7.55± 0.65	13.63± 13.85	711.33± 384.97	9.46± 2.39	133.5± 3.46	102±1 .77
Medi-um-dose group	5.4± 1.44	0.48± 0.08	1.93± 0.41	1.32± 1.37	57.62± 4.63	33.49± 2.15	50.2± 22.45	115.7± 21.3	124.6± 32.1	8.84± 1.9	13.3± 4.5	516.4± 207.37	9.71± 1.77	133.7± 3.43	99.3± 3.53
High-dose group	4.65± 1.69	0.51± 0.15	1.85± 0.46	2.87± 4.99	58.83± 5.35	34.61± 2.8	42.56± 5.48	112.89± 20.04	123.33± 36.2	7.22± 1.68	10.78± 7.51	584.22± 212.24	9.34± 2.21	134.11± 3.26	100.11± 1.54

*Note: n=10, half male and half female, compared with the blank control group: in the medium-dose group TBIL: P=0.03<0.05, ALP: P=0.018<0.05, CK: P=0.034<0.05, TG: P=0.021<0.05=The rest of the P>0.05.

Table 10: Effect of percolation of the fruit on serum biochemical indexes after the period of convalescence in rats.

Groups	GLU (m mol/L)	TG (m mol/L)	CHOL (m mol/L)	TBIL (u mol/L)	TP (g/L)	ALB (g/L)	ALT (U/L)	AST (U/L)	ALP (U/L)	BUN (m mol/L)	CRE (u mol/L)	CK (U/L)	K^+ (m mol/L)	Na^+ (m mol/L)	Cl^- (m mol/L)
The blank group	5.71± 1.97	0.65± 0.21	2.42± 0.97	12.1± 10.64	60.38± 8.18	36.03± 5.36	43.38± 11.21	173.13± 41.03	87± 49.26	7.89± 0.92	6.5± 18.42	1646.5± 1859.77	10.67± 2.71	131.75± 3.99	101± 2.88
Low-dose group	9.57± 3.08	0.58± 0.15	2.7± 0.41	12.24± 6.28	64.77± 5.32	36.83± 3.1	80.11± 35.07	242.56± 97.68	138.89± 38.04	7.45± 0.56	2.22± 6.87	1188.89± 979.69	10.84± 4.65	134± 5.15	101.56± 1.33
Medi-um-dose group	8.7± 2.68	0.7± 0.23	2.85± 1.18	12.04± 11.15	63.2± 5.45	35.84± 4.13	120.63± 98.77	408.25± 352.65	141.78± 42.52	8.84± 2.1	3.33± 13.43	3174.5± 4554.8	9.53± 3.17	134.56± 3.94	103.56± 1.33
High-dose group	8.64± 2.68	0.6± 0.12	2.26± 0.48	5.98± 3.73	61.77± 3.71	34.45± 1.9	70.6± 27.79	275.1± 270.14	124.8± 39.22	7.62± 1.58	9.1± 3.11	3040.89± 4541.79	8.86± 2.7	135.5± 3.6	102.6± 1.51

*Note: n=10, half male and half female, compared with the blank control group: in the Low -dose group, GLU: P=0.005<0.05; in the medium-dose group: ALT: P=0.004<0.05, AST: P=0.008<0.05; The rest of the P>0.05.

Histopathological examination

Autopsy overview: At the end of drug delivery and the end of convalescence observation, the rats in each group were dissected and observed by naked eyes according to the plan: the skin of the rats in each group was intact and skin lesions such as depilation redness and swelling were not observed. There was not secretion or trauma in natural orifice such as ear, mouth, nose and anus; There were not cyanosis, yellow and red spots in the eyes; The subcutaneous tissue was free of bleeding and masses, and the muscle was pink, shiny and elastic. There was not fluid or gas retention and not odor in chest cavity abdomen cavity and skull cavity. The position, shape, texture and color of various organs were normal and there was not adhesion or other abnormal changes.

Table 11: Effect of percolation of the fruit on viscera coefficient after 4-weeks drug delivery in rats (male) (x ± s, g/100g).

Groups	Heart	Liver	Spleen	Lung	Kidney	Brain	Thymus	Testis	Epididymis	Prostate
The blank group	0.32±0.02	2.52±0.19	0.19±0.03	0.43±0.02	0.67±0.06	0.53±0.11	0.18±0.04	1.03±0.12	0.12±0.01	0.25±0.11
Low-dose group	0.31±0.01	2.60±0.03	0.21±0.02	0.48±0.07	0.68±0.05	0.67±0.07	0.20±0.02	0.78±0.52	0.07±0.06	0.29±0.11
Medi-um-dose group	0.33±0.02	3.01±0.43	0.20±0.02	0.52±0.05	0.78±0.11	0.67±0.14	0.16±0.05	1.17±0.03	0.07±0.06	0.26±0.04
High-dose group	0.33±0.02	2.33±0.40	0.26±0.01	0.52±0.09	070±0.06	0.48±0.11	0.15±0.03	0.83±0.02	1.07±0.08	0.13±0.03

*Note: n=10, half male and half female, compared with the blank control group: in the Low -dose group, GLU: P=0.005<0.05; in the medium-dose group: ALT: P=0.004<0.05, AST: P=0.008<0.05; The rest of the P>0.05.

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Viscera coefficient: After 4-weeks drug delivery and 2-weeks convalescence, the viscera coefficient of the rats in the low-dose, medium-dose and high-dose groups were all within the normal range, except for individual indicators of individual dose groups, with no statistical significance compared with the control group as shown in Table 11- 14.

Table 12: Effect of percolation of the fruit on viscera coefficient after 4-weeks drug delivery in rats (female) (x ± s, g/100g).

Groups	Heart	Liver	Spleen	Lung	Kidney	Brain	Thymus
The blank group	0.34±0.03	2.82±0.33	0.21±0.02	0.55±0.09	0.69±0.03	0.91±0.29	0.21±0.02
Low-dose group	0.36±0,06	2.80±0.24	0.23±0.03	0.52±0.07	0.73±0.07	0.73±0.07	0.16±0.02
Medium-dose group	0.33±0.02	2.33±0.41	0.17±0.02	0.91±0.89	0.7±0.06	0.48±0.11	0.15±0.03
High-dose group	0.35±0.01	2.65±0.21	0.22±0.02	0.53±0.05	0.7±0.02	0.81±0.09	0.17±0.05

*Note: n=10 in each group. Compared with the blank control group: in the low-dose group, spleen P=0.02<0.05. In the medium-dose group, spleen P=0.016<0.05; In the high-dose group: liver P=0.016<0.05; The rest of the P > 0.05.

Table 13: Effect of percolation of the fruit on viscera coefficient after the period of convalescence in rats (male) (x ± s, g/100g).

Groups	Heart	Liver	Spleen	Lung	Kidney	Brain	Thymus	Prostate	Testis	Epididymis
The blank group	0.47±0.24	3.02±0.35	0.21±0.01	0.43±0.04	0.38±0.03	0.58±0.06	0.15±0.04	0.28±0.02	0.48±0.03	0.07±0.01
Low-dose group	0.31±0.05	2.87±0.31	0.22±0.03	0.45±0.03	0.35±0.04	0.47±0.06	0.15±0.03	0.31±0.05	0.51±0.01	0.08±0.01
Medi-um-dose group	0.32±0.04	2.81±0.15	0.23±0.04	0.45±0.07	0.34±0.02	0.66±0.05	0.16±0.01	0.37±0.25	0.47±0.04	0.08±0.02
High-dose group	0.31±0.02	2.99±0.2	0.19±0.01	0.42±0.03	0.36±0.03	0.47±0.12	0.14±0.04	0.31±0.05	0.48±0.02	0.08±0.01

*Note: n=10 in each group. Compared with the blank control group: in the low-dose group, heart P<0.05; In the medium-dose group: heart P<0.05; The rest of the P > 0.05.

Table 14: Effect of percolation of the fruit on viscera coefficient after the period of convalescence in rats (female) (x ± s, g/100g).

Groups	Heart	Liver	Spleen	Lung	Brain	Thymus	Kidney
The blank group	0.39±0.03	3.19±0.27	0.24±0.03	0.47±0.11	0.71±0.12	0.19±0.02	0.39±0.03
Low-dose group	0.34±0.03	2.87±0.28	0.21±0.02	0.54±0.06	0.67±0.12	0.19±0.04	0.38±0.03
Medium-dose group	0.34±0.05	3±0.21	0.25±0.03	0.54±0.08	0.72±0.13	0.16±0.09	0.37±0.0
High-dose group	0.36±0.06	2.27±1.35	0.24±0.04	0.55±0.18	0.76±0.1	0.19±0.06	0.35±0.05

*Note: n=10 in each group. Compared with the blank control group: in the low-dose group, heart P<0.05; In the medium-dose group: heart P<0.05; The rest of the P > 0.05.

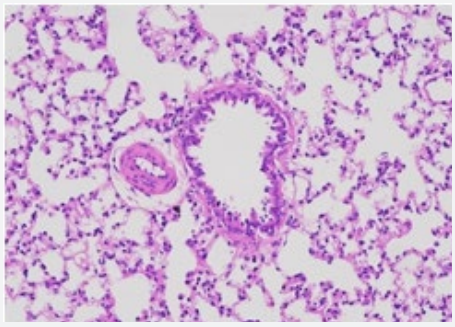

Figure 5: Lung tissue sections of the blank group 200X.

Effect on pathological morphology of important organs and tissues: Compared with the blank control group during the drug delivery period, liver room dilation was observed in 1 male rat in the high-dose group, lymphocyte infiltration was observed in 1 female rat in the medium-dose group, and thymus gland hyperplasia was observed in 1 male rat in the low-dose group and 1 male rat in the medium-dose group. Significant abnormalities weren't found in other tissues. In the convalescence period, compared with the blank control group, the small U in the center of the liver of 1 male rat in the high-dose group was widened, and lung congestion was found in 1 male rat in the medium dose group, and obvious abnormality wasn't found in other tissues. Due to the lack of drug dose dependence in these pathological manifestations, and the difference between each dose group and the control group was not statistically significant, it was considered that it had nothing to do with the toxic effect of the fruit, considering that it was caused by their own diseases of rats or other reasons [24]. Significant abnormal changes weren't observed in other organs. Combined with the above results of organ quality and organ coefficient it showed that continuous drug delivery for 4 weeks didn't have significant effect on the tissue morphology and structure of rats'organs Figure 5-10.

Conclusion and Discussion

In the anti-virus *in vitro* experiment, the percolation of the fruit was used as antivirus liquid, hich showed a good inhibitory effect on RSV, EV-71 and HSV-1. Polyphenols were selected as the antiviral active parts of the fruit, and the first column volume eluted by D101 macro-porous resin with 25% ethanol showed good efficacy TI was 200.85). The results showed that the polyphenols

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of the fruit had a good inhibitory effect on HSV-1 virus. At present, international anti-HSV-1 virus mainly consists of some antibiotics [25]. Traditional Chinese medicine has the advantages of wide spectrum antiviral less side effects and so on, which is one reason why traditional Chinese medicine is widely used. In the following study the antiviral mechanism of percolation of the fruit will be further explored. With the further research, the specific mechanism of antiviral action would be elucidated, and effective monomers would be separated. The long-term toxicity test of rats showed that the percolation of the fruit was continuously given for 4 weeks, and poisoning symptoms weren't observed in the rats. Body weight, food intake, hematology index examination, serum biochemistry, organ quality and organ coefficient were not statistically significant compared with the control group, and all were within the normal range. After anatomical and histological observation, histopathological changes weren't found in the heart, liver, spleen, lung, kidney, ovary, testicle and other organs of the rats, except for slight degeneration of liver, spleen and heart that was not related to drug use factors in some rats. After 2-weeks convalescence period, rats were in the obvious health state. And there was not statistically significant difference in the effect of different doses of purple leaf plum fruit on the indexes of rats compared with the control group. The experimental study showed that there was not obvious toxic reaction or delayed toxic reaction in the percolation of the fruit and it was safe to take large doses for a long time which provided experimental basis for clinical application. In conclusion, the fruit has a good prospect of antiviral application and a guarantee of safety and non-toxicity which is of great development and utilization value.

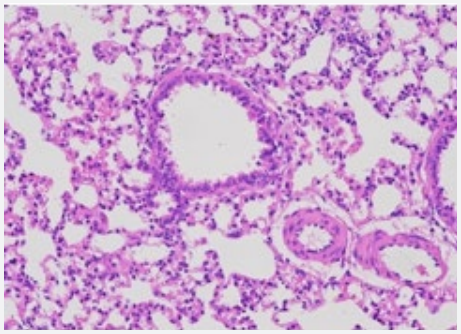

Figure 6: Lung tissue sections of the high-dose group 200X.

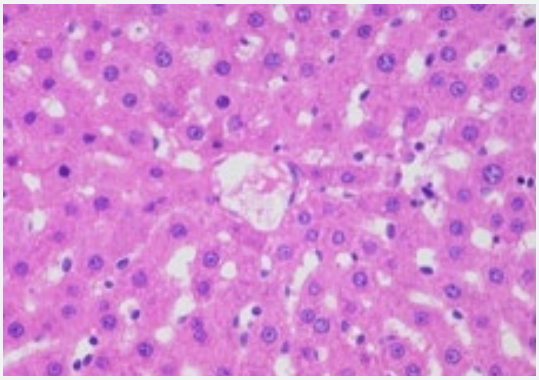

Figure 7: A liver tissue sections of the blank group 400X.

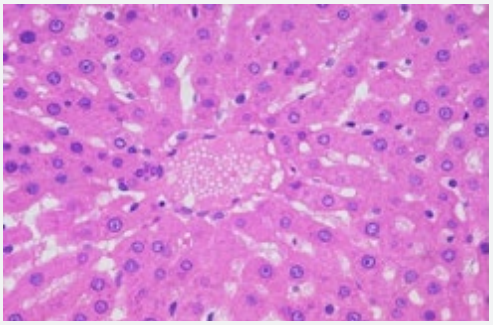

Figure 8: Liver tissue sections of the medium dose group 400X.

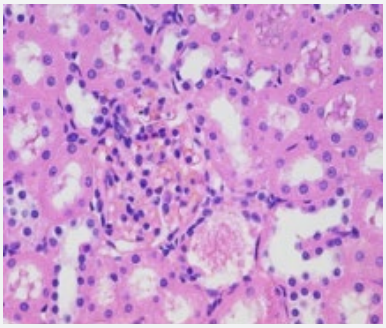

Figure 9: Renal tissue sections of the blank group 400X.

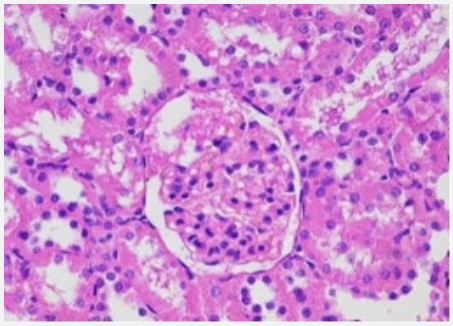

Figure 10: renal tissue sections of the low-dose group 400X.

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