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# Safety Evaluation of Andrographolide-nanosuspensions

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## ABSTRACT

**Background:** *Andrographolide* (ANDRO) is a hydrophobic drug, which faces the problem of limited absorption due to poor water solubility. The current research prepared *andrographolide* nanosuspensions (ANDRO-NS) and examined *in vivo* toxicity for mice.

**Methods:** ANDRO-NS were prepared by anti-solvent precipitation method, transmission electron micrographs, granularity analysis and *in vitro* release were used to characterize the ANDRO-NS, we evaluate the safety of the ANDRO-NS by using the acute toxicity test, local irritation test and chronic toxicity test.

**Result:** The particle size of ANDRO-NS was (568.51±13.74 nm). The $LD_{50}$ for ANDRO-NS was 548.91 mg/kg after oral administration to KM mice with a 95% CI of 468.19-645.03 mg/kg. The white blood cell counts and hemoglobin levels for the experimental groups were lower than controls receiving only saline. Serum aspartate transaminase, creatinine and blood urea nitrogen levels were greater than controls after 7 and 14 days of once-daily administration. After 14 days of administration, the platelet counts as well asalanine transaminase levels were, in addition, Histological observations indicated that interstitial kidney tissues were wider than controls and showed episodic bleeding after 7 days of administration. The highest dose administered also resulted in the dilation and blood engorgement of the central hepatic veins with some severed hepatic cords. Mice receiving the lowest dose of ANDRO-NS we administered appeared healthy and similar to controls receiving saline only. Following 14 days of administration, we found significant vacuolar degeneration of renal tubular epithelial cells and glomerular atrophy for the high-dose group as well as edema and necrosis in liver cells. The medium-dose group displayed kidney interstitial tissue widening with scattered bleeding, inflammatory cell infiltration and hepatocyte edema. The low-dose group displayed dilated renal tubules and irregularly-arranged liver cells as well as bleeding in the hepatic sinusoids. Therefore, short-term administration of andrographolide suspensions resulted in inflammation and time- and dose-dependent toxic effects on the kidneys and liver.


**Key words:** *Andrographolide*, Anti-solvent precipitation method, Chronic toxicity, Nanosuspensions, Safety evaluation.

## INTRODUCTION

*Andrographolide* (ANDRO) is the primary bioactive ingredient derived from the Acanthaceae family member the green chiretta (Andrographis paniculata) and is used in Chinese medicine. ANDRO is a naturally occurring bicyclic diterpenoid whose other members include the bioactive compounds retinol, phytol and forskolin. ANDRO has been shown to have anti-inflammatory (Xu *et al.,* 2019), anti-infective (Zhang *et al.,* 2020), anti-cancer (Ahiwale *et al.*, 2020), anti-hyperglycemic (Liang, 2014) and anti-angiogenic properties (Guo *et al.,* 2018) and can function as an immune stimulator (Kang *et al.,* 2020) and possesses anti-reproductive and other pharmacological effects (Yang *et al.*, 2019). However ANDRO is not water soluble, shows poor oral absorption with low bioavailability and is chemically unstable in body fluids (Zhang *et al.,* 2019; Guo *et al.,* 2019). These have limited its wide clinical application.

Nano-suspensions take advantage of the stabilizing effect of surfactants to disperse drug particles in water *via* stable nano-colloidal dispersions formed through crushing or controlled crystallization technologies. Nanoparticles enhance the solubility and dissolution rate of poorly soluble drugs and can increase bioavailability (Prasad *et al.,* 2019; Yin *et al.,* 2018). In the present study, we applied anti-solvent recrystallization technology to prepare andrographolide nano-suspensions (ANDRO-NS) that significantly improved andrographolide dissolution in comparison with the raw materials. However, these suspensions were accompanied by toxic side effects in animals. We therefore performed a safety evaluation of ANDRO-NS and conducted acute and subacute toxicity tests in mice as an initial evaluation to provide a theoretical basis for the further use of this formulation.

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## MATERIALS AND METHODS

### Ethics statement

Animal experiments were performed in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

### Instruments and equipment

Nanoparticles constructed in this study were measured using a BT-9300ST laser particle size distribution analyzer (Bettersize Instruments, Shanghai, China) and a JEM2100 transmission electron microscope (TEM) (JEOL, Tokyo, Japan).

### Materials

*Andrographolide* (≥ 95%) was obtained from Henan Shengtai Biotechnology (Zhengzhou, China). Poloxamer 188 was obtained from BASF (Manheim, Germany). Tween 80, sodium lauryl sulfate (SDS) and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent (Shanghai, China).

### Animals

A total of seventy (n=100) twenty-eight-days old healthy KM mice (initial weight 20±2 g) were purchased from Experimental Animal Center, Zhengzhou University (Zhengzhou, China). Moreover, the proportion of the quantity of male and female in each group was 1:1 to decrease the influence of sex. The mice self-propagated through the experimental animal center and possessed a similar genetic background. All the screened mice were subjected to the same immunization program and were determined to be free of other diseases before the experiment.

### Preparation of andrographolide nanoparticles

ANDRO-NS were prepared using 0.025 g poloxamer 188 and 0.5 mL Tween 80 added together to 50 mL distilled water (dissolution medium) under stirring until complete dissolution. Andrographolide (0.4 g) was dissolved in 10 mL DMSO and was added dropwise to the detergent solution at 25°C using a stirring rate of 1400 rpm for 60 min. The solution was then filtered through a 0.8 μm microporous membrane of Nylon66 (TAILIN Bioengineering Co., LTD, ZheJiang).

### Quality evaluation of ANDRO-NS

The average particle size of ANDRO-NS was determined using a laser particle size analyzer (see above) and the particles were also visualized using TEM. In brief, freshly prepared andrographolide suspensions was added dropwise onto copper grids and allowed to stand for 2-3 min. then stained with 2% phosphotungstic acid for 2-3 min.

The andrographolide raw material and andrographolide suspensions equivalent to 40 mg andrographolide were dialyzed (molecular weight cut-off 13000) against dissolution medium (pH 6.8) at 37°C with stirring at 100 rpm. Samples (5 mL) were taken at 10, 20, 30, 60, 120 and 180 min and each time an equivalent volume of dissolution medium was added back. The samples were centrifuged at 14000 rpm for 10 min and analyzed using liquid chromatography with an Agilent TC-C18 column (250 × 4.6 mm, 5 μm) (Agilent, Santa Clara, CA ,USA). The mobile phase was methanol / water (65/35 v/v) at 1.0 mL/min using a detection wavelength of 225 nm and an injection volume of 20 μL and an ambient column temperature.

### Determination of $LD_{50}$

The lethality of the ANDRO-NS preparation was initially examined in preliminary experiment using mice that had been fastest for 12 h and allowed water only 2 h prior to the experiments. Mice (20) were randomly divided into 5 groups and given different doses of ANDRO-NS preparation intragastrically. The mice were then returned to their normal diet and observed for signs of morbidity and mortally over a 24 h period. The absolute lethal and the maximum tolerated dosages were calculated as previously described (Wu *et al.*, 2017).

The results of the preliminary test were used to establish formal tests using 40 mice randomly divided into 5 groups under the conditions described above. The dose ratio between groups used was 1: 0.8 and the ANDRO-NS suspensions were given by oral gavage. The mice were returned to their normal routine of feeding and watering and were observed continuously for 5 d for symptoms, physiological state and deaths at regular intervals. Based on the number of deaths in each group, the half lethal dose ($LD_{50}$) using the modified Kor's calculation method was determined (Pan *et al.,* 2012).

### Subchronic toxicity test

Healthy mice (36) with uniform body weight were randomly divided into 4 groups and divided into 3 dosage groups according to the $LD_{50}$ and were given ANDRO-NS at high, medium and low levels (50, 25 and 5 mg/kg, respectively). The control group was given an equivalent volume of saline by gavage. The drug was administered once a day at 0.5 mL dose for 14 days. The health status, activity, eating, drinking, excrement, illness and death of the mice were observed and recorded daily. Blood was collected on days 8 and 15 from four mice *via* retro-orbital puncture. The mice were then selected for euthanasia in each group. Afterwards, excising the gut from abdominal cavity and stripping off the mesentery using sterilized surgical knife and the livers and kidneys were removed and stored in 10% formaldehyde (formalin).

### Physiological and biochemical tests

Blood samples were examined for red blood cells (RBC) and white blood cells (WBC), hematocrit (HCT) and hemoglobin (HGB). Serum was examined for levels of alanine transferase (ALT), glutamic oxaloacetylase (AST), creatinine (CRE) and urea nitrogen (UREA).

### Histopathological observation

The tissue samples were prepared as previously described and stained with haematoxylin and eosin (H&E). Tissue morphology was observed by light microscopy.

### Statistical analyses

SAS 18.0 (IBM, Chicago, Ill, USA) was used to analyze the data. One-way analysis of variance was used for the blood physiological and biochemical indexes and multiple comparisons were made based on the LSD method. $P<0.05$

indicated a significant differences and results were expressed as mean±standard deviation (SD).

## RESULTS AND DISCUSSION

### Preparation of ANDRO-NS

The particle size distribution of the nanoparticles indicated that the preparation was uniform and a single peak was detected using laser light scattering. The average particle size was 568.51±13.74 nm (Fig 1). The nanoparticles appeared round or oval and uniform in shape under TEM (Fig 2). These results indicated that the prepared nanoparticles have a small and stable grain size. The concentration and type of stabilizer have an important impact on the particle size of the suspension. (Bhavna and Ali, 2014; Fujimura *et al.,* 2016). In order to ensure the stability of the suspension, compound stabilizers are often used in production. When Poloxamer 188 or Tween-80 were used alone, the andrographolide nanosuspensions produced were unstable and the crystals began to aggregate and became larger when left for 24 hours. Therefore, in this experiment, Poloxamer 188 and Tween 80 were used as surfactants, which may be because both are non-ionic surfactants that maintain the stability of the suspension by creating steric hindrance.

### *In vitro* release test results of ANDRO-NS

Conversion of the andrographolide to ANDRO-NS significantly improved its solubility and at a dissolution time of 180 min, the level of ANDRO-NS was 2.43 times that of andrographolide (Fig 3). This indicated that our preparation method effectively increased the specific surface area of andrographolide. The release curves for andrographolide and ANDRO-NS were fitted with the Weibull model *in vitro* release and the equations was y = 0.010 x + 0.145 (r=0.900) and y=0.027 x + 0.310 (r=0.922).

### Acute toxicology test results

The initial *in vivo* experiments were meant to assess whether mice could tolerate extreme doses of ANDRO-NS. Mice in the high-dose group (1200 mg/kg) showed mental excitement after being administered by gavage for 1-2 minutes, then they wandered, ran, jumpedÿand eventually fell to the ground in full-body convulsions. The mice began to die after about 5 minutes. Mice in the 251.65 mg/kg dose group developed mild neurological symptoms with slight muscle twitches and gathered themselves into a pile. After about 2 hours, they returned to normal state without death. They appeared completely normal after one day. These observations indicated that the median lethal dose ($LD_{50}$) was 548.91 mg/kg with a 95% CI of 468.19-645.03 mg/kg (Table 1). Researchers have found through literature studies that $LD_{50}$ of andrographolide has a wide dynamic range from 500 to 2000 mg/kg (Chen *et al.,* 2005). They also observed that andrographolide had no obvious effect on toxicity by nanosuspension.

### Subacute toxicity test results

This study modified the dosages based on the acute toxicity test results and administered ANDRO-NS at 50, 25 and 5 mg/kg for seven days. In all test groups, white blood cells (WBC) and hemoglobin (HGB) were significantly lower than

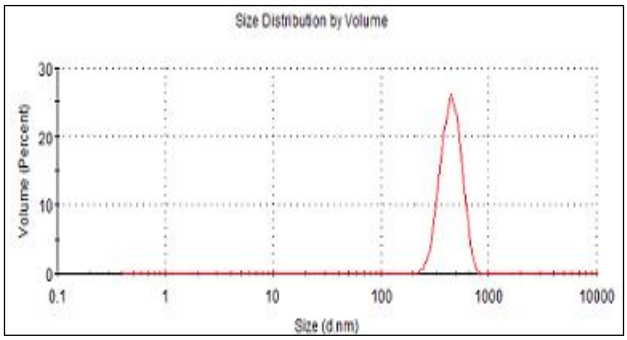


**Fig 1:** Particle size distribution of ANDRO-NS.

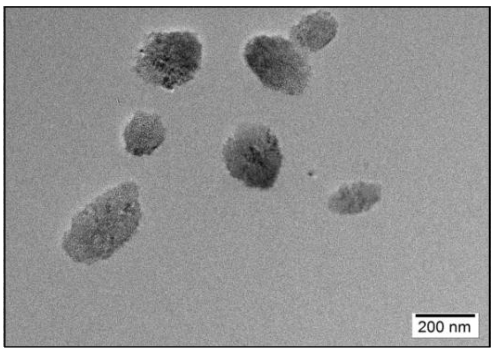


**Fig 2:** Transmission electron micrographs of ANDRO-NS (× 6000).

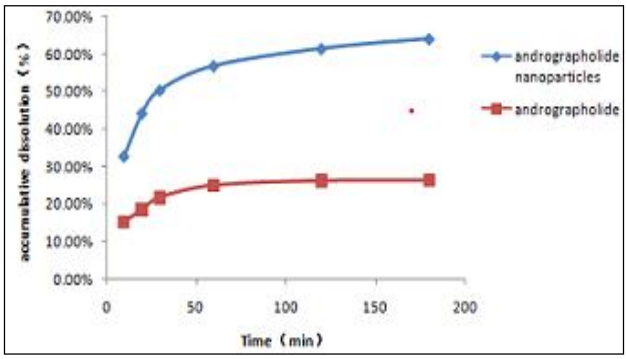


**Fig 3:** Dissolution curves for ANDRO-NS and ANDRO.

**Table 1:** Acute toxicity tests in mice using ANDRO-NS.

| †mg/kg | Numbers | Deaths | Mortality / % |
|---|---|---|---|
| 1200 | 10 | 10 | 100% |
| 960 | 10 | 7 | 70% |
| 768 | 10 | 6 | 60% |
| 614.4 | 10 | 6 | 60% |
| 491.52 | 10 | 5 | 50% |
| 393.16 | 10 | 4 | 40% |
| 314.57 | 10 | 2 | 20% |
| 251.65 | 10 | 0 | 0 |

those in the control group (P<0.05). The red blood cells (RBC) in the high-dose group decreased by 26.40%, reaching the level of statistical significance (P<0.05). Interestingly, the platelet (PLT) count in the high- and medium-dose groups was significantly higher than in the control group (11.42% and 9.80%, respectively). In comparison between the test groups, the levels of RBC and HGB in the high-dose group were notably lower than those in the medium- and low-dose groups (P<0.05). In addition, PLT counts for the high- and medium-dose groups were remarkably higher than those in the low-dose group (P<0.05).

After fourteen days of ANDRO-NS administration, the WBC counts in each test group became less than the control group (P<0.05), while the numbers for RBC, HGB and PLT in the high- and medium-dose groups increased comparing to the control group (P<0.05). Set test groups side by side, the rate of RBC, HGB and PLT s in each group were significantly higher than those in the low-dose group (P<0.05). Overall, WBC, RBC, PLT and HGB levels for each test group increased after fourteen days (P<0.05) (Table 2).

The serum biochemical blood test of mice in the high dosage group showed that after seven days of ANDRO-NS administration, aspartate aminotransferase (AST), creatinine (CRE) and blood urea nitrogen (BUN) grew by 43.68%, 25.47% and 54.61% compared to the control group while reaching the statistical significance (P<0.05). AST, CRE and BUN for the low-dose group were also higher than those of the control group, but the differences were not significant (P>0.05). AST, ALT, CRE and BUN levels in the high-dose group were greater than middle- and low-dose groups. In contrast, AST and BUN for the high-dose group were 41.17% and 31.31% than low-dose groups, respectively and significant (P<0.05). After fourteen days of ANDRO-NS administration, the numbers of AST, ALT, CRE and BUN for each test group were significantly higher than the control groups (P<0.05). The CRE level in the high-dose group was 2.23 times higher than that of the control group, while the BUN level was 3.33 timer higher. The comparison between test groups indicated that AST, ALT, CRE and BUN in the high-dose group were much higher than those in the medium- and low-dose groups (P<0.05). AST, ALT, CRE and BUN in the blood of the high-dose group and the medium-dose group increased significantly after 14 days of administration compared with the 7-day levels (Table 3). Blood physiological and biochemical indicators are important to evaluate the pathology of the body, tissues and organs (Wlaź *et al*., 2015). Alanine transferase (ALT) and alkaline phosphatase (ALKP) are used to determine whether the liver is damaged, while CRE and BUN can indicate whether the kidney is damaged (Al- Batran *et al*., 2013). Hu *et al* (2010) revealed that the levels of ketamine (KET), erythrocytes (ERY), BUN, CRE, ALP, lactate dehydrogenase (LDH) and N-acetyl-glucoseaminidase (NAG) in the urine were increased in the high-dose Lianbizhi (andrographolide) Injections treated rats, we observed that the RBC, HGB, PLT, AST, ALT, CREA and BUN levels of high-dose and medium-dose groups were higher than that of the control group after 14 days of administration.

**Table 2:** Effects of ANDRO-NS on blood physiological indexes in mice.

| Time | Tests | Control | Low dose | Medium dose | High dose |
|---|---|---|---|---|---|
| 7d | White blood cell count $10^9$/L | 90.1±2.68$^a$ | 78.55±6.29$^b$ | 74.5±6.36$^b$ | 71.5±4.95$^b$ |
| | Red blood cell count $10^{12}$/L | 4.81±0.32$^a$ | 4.48±0.78$^a$ | 4.24±0.56$^a$ | 3.53±0.71$^b$ |
| | Hemoglobin g/L | 100.5±3.53$^a$ | 94.5±2.12b | 73.5±3.19$^c$ | 62.5±3.53$^d$ |
| | Platelet $10^9$/L | 3082.00±19.09$^b$ | 3131.00±26.87$^b$ | 3384.00±77.07$^a$ | 3434.00±15.55$^a$ |
| 14d | White blood cell count $10^9$/L | 100.65±3.74$^a$ | 92.80±3.95$^b$ | 88.40±6.22$^b$ | 78.55±3.60$^c$ |
| | Red blood cell count $10^{12}$/L | 4.65±0.21$^b$ | 4.64±0.36$^b$ | 5.41±0.26$^a$ | 5.22±0.67$^a$ |
| | Hemoglobin g/L | 98.01±4.24$^c$ | 98.50±2.12$^c$ | 108.40±1.97$^a$ | 104.00±1.41$^b$ |
| | Platelet $10^9$/L | 3505.00±118.08$^c$ | 3525.6±127.98$^b$ | 3571.00±103.12$^b$ | 3721.50±51.61$^a$ |

In the same row, values with different small letter superscripts indicate significant differences (P<0.05) and with the same or no letter superscripts no significant difference (P>0.05).

**Table 3:** Effects of ANDRO-NS on mouse blood chemistries.

| Time | Tests | Control | Low dose | Medium dose | High dose |
|---|---|---|---|---|---|
| 7d | Glutamic oxaloacetylase (U/L) | 78.14±4.73$^c$ | 79.53±7.38$^c$ | 95.70±7.93$^b$ | 112.27±7.53$^a$ |
| | Alanine aminotransferase (U/L) | 51.83±9.68$^a$ | 46.67±7.23$^a$ | 51.84±10.22$^a$ | 59.05±8.87$^a$ |
| | creatinine (μmol/L) | 5.81±1.09$^a$ | 6.28±1.42$^a$ | 6.62±1.57$^a$ | 7.29±1.22$^a$ |
| | Urea nitrogen (μmol/L) | 4.34±0.81$^b$ | 5.11±1.08$^b$ | 6.63±1.12$^a$ | 6.71±1.12$^a$ |
| 14d | Glutamic oxaloacetylase (U/L) | 80.85±6.50$^c$ | 90.97±11.50$^b$ | 103.87±8.39$^b$ | 121.43±14.84$^a$ |
| | Alanine aminotransferase (U/L) | 57.03±4.82$^c$ | 66.08±9.19$^b$ | 74.70±7.33$^b$ | 89.33±8.32$^a$ |
| | creatinine (μmol/L) | 5.65±1.41$^d$ | 7.21±1.67$^c$ | 10.96±1.55$^b$ | 12.62±1.64$^a$ |
| | Urea nitrogen (μmol/L) | 4.71±1.61$^d$ | 6.35±2.72$^c$ | 10.49±2.89$^b$ | 15.68±3.79$^a$ |

In the same row, values with different small letter superscripts indicate significant differences (P<0.05) and with the same or no letter superscripts no significant difference (P>0.05).

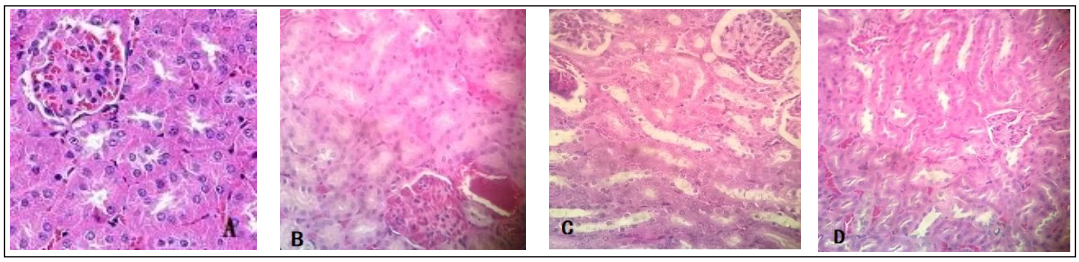


**Fig 4:** Kidney histological sections after 7 days of ANDRO-NS administration.
A. Control B. Low dose C. Medium dose D. High dose. H&E staining × 100.

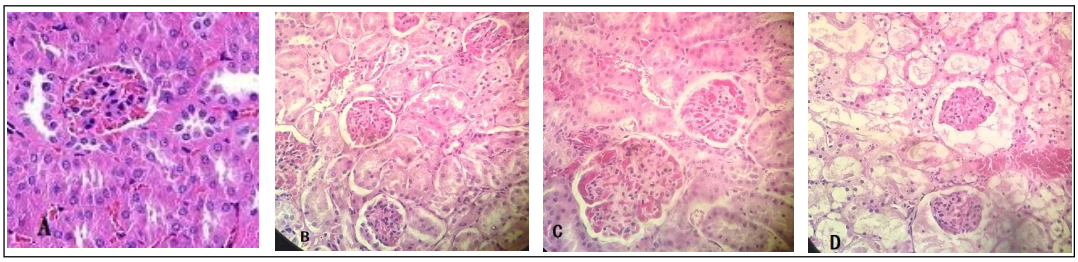


**Fig 5:** Kidney histological sections after 14 days of ANDRO-NS administration.
A. Control B. Low dose C. Medium dose D. High dose. H&E staining × 100.

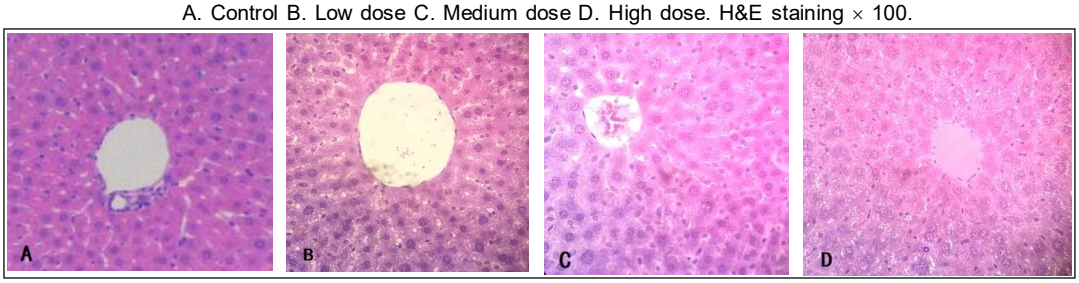


**Fig 6:** Liver histological sections after 7 days of ANDRO-NS administration.
A. Control B. Low dose C. Medium dose D. High dose. H&E staining × 100.

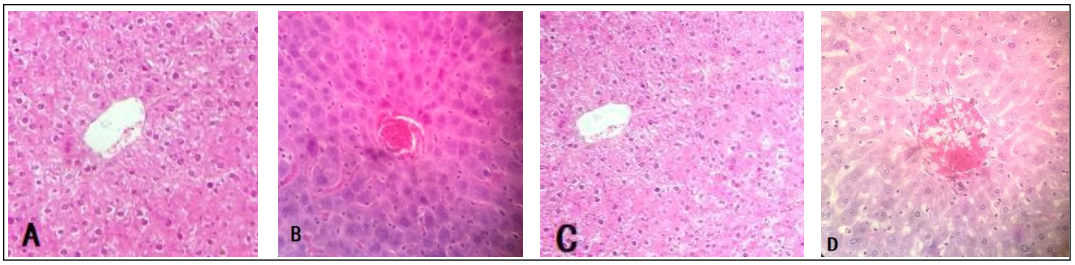


**Fig 7:** Liver histological sections after 14 days of ANDRO-NS administration.
A. Control B. Low dose C. Medium dose D. High dose. H&E staining × 100.

We also examined the histology of the kidneys and livers of the mice in the three test groups compared with the control group seven days and fourteen days after the ANDRO-NS administration. The renal interstitium for the high-dose group showed signs of hemorrhage. Compared with the control group, the renal interstitium of the mice in the high-dose and medium-dose groups was wide (Fig 4C and D). There was also sporadic hemorrhaging in the renal interstitium of the low-dose group (Fig 4B). Fourteen days after the ANDRO-NS administration, compared with the control group (Fig 5A), the high-dose group exhibited renal glomerulus atrophy, vacuolar degeneration of renal tubular epithelial cells, sporadic bleeding and inflammatory cell infiltration (Fig 5D). The renal tubules in the low-dose group were also dilated (Fig 5B).

After seven days of the experiment, the liver examination showed that the central hepatic veins of the high-dose group were dilated and the hepatocyte cords were disconnected (Fig 6D). Hepatocytes of the medium-dose group were loosened (Fig 6C). These changes were not seen in the low-dose group and the hepatocyte cords remained intact. The sinusoidal bleeding, necrosis and other phenomena were absent compared to controls (Fig 6A and B). After fourteen days of the administration, compared with the control group (Fig 7A), the hepatocellular edema and hepatic necrosis occurred in the high-dose group (Fig 7D). There were signs of hepatocyte edema and hepatic sinusoidal degeneration in the medium-dose group (Fig 7C). In the low-dose group, hepatocytes were arranged irregularly, hepatic sinusoids were irregular and some bleeding

symptoms were observed (Fig 7B). Although, it is generally known that andrographolide can cause nephrotoxicity (Lu *et al.,* 2010; Lu *et al.,* 2011). However, these types of livers damage were more serious than reported in the literature (Fang *et al.,* 2007). This phenomenon indicates that the andrographolide prepared by the study, caused an inflammatory reaction and fourteen days of high-dose administration resulted in hepatocyte edema and liver cell necrosis. The toxicity mechanism for ANDRO-NS needs further study.

## CONCLUSION

We prepared ANDRO-NS with an average particle size of 568.51±13.74 nm, the $LD_{50}$ for ANDRO-NS was 548.91 mg/kg which indicating a lowly poisonous. We determined that high levels of ANDRO-NS administered over a short period of time caused inflammation and toxic effects on the kidney and liver. The drug toxicity was dose-dependent and indicates that experiments using the ANDRO-NS preparation should be closely observed and the dosage strictly controlled.

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