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Safety investigation on total steroid saponins extracts from *Dioscorea zingiberensis* C.H. Wright: Sub-acute and chronic toxicity studies on dogs

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Abstract

Sub-acute and chronic toxic effects of total steroidal saponins (TSSN) extracts from *Dioscorea zingiberensis* C.H. Wright on various internal organs and biochemical indicators have never been studied before and this study is the first of its kind to demonstrate sub-acute and chronic toxicities of TSSN on dogs. Administration of TSSN extracts at doses up to 3000 mg/Kg daily for 14 days, no biochemical and organ changes were observed on the experimental groups of dogs. Further, chronic toxicity study through oral administration of TSSN extracts at the gradual doses of 50, 250 and 500 mg/Kg for 90 days followed by a 2-week recovery assay revealed absence of significant architectural and morphological changes in internal organs which were confirmed through histopathological examination and merely no significant alteration in the biochemical indicators including hematologic and urine analysis and electrocardiogram compared to the control dogs. This toxicological evaluation came across with the finding that the herbal preparation can be considered as nontoxic and animals could tolerate the extracts at doses up to 500 mg/Kg with LD₅₀ greater than 3000 mg/Kg. It may serve as a preliminary scientific evidence for further therapeutic investigations.

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Conflicts of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Graphical abstract

Keywords

Dioscorea zingiberensis C.H. Wright; Steroid saponins; Sub-acute toxicity; Chronic toxicity

1. Introduction

Traditional Chinese Medicine (TCM) or plant-derived natural products such as herbal preparations, natural remedies and drugs containing a natural compound have been utilized in medication of human health and amelioration of illnesses for a long period of time [1]. Being used as an alternative means of disease prevention and treatment, TCM or their herbal products have remained attractive to doctors and patients owing to their moderate therapeutic effects and minimal side effects. Nowadays many herbal remedies, coming as an equivalent therapeutic choice to their synthetic counterparts, claimed to have lesser adverse reactions up on long term use in some refractory health disorders such as cardiovascular and cancer diseases [2, 3]. Western drugs are said to have severe adverse reactions equally to their desirable therapeutic effects. However, the natural alternatives do have comparable toxicities attributed to the synergetic or antagonistic interactions among their bioactive constituents such as saponins, flavonoids and alkaloids [4, 5]. There are a number of scientific reports on the toxicity of TCM [6, 7]. Along with an increasing consumption around the world, the safety of TCM and their herbal products has become a major concern and account more than their quality and efficacy.

Dioscorea zingiberensis C.H. Wright (*D. zingiberensis*) also named as Huangjiang (黄姜 in Chinese) belongs to Dioscoraceae family and *Dioscorea* genus which is uniquely and widely distributed in China [8]. It is a perennial grass vine plant with a characteristic cool nature and a little bit bitter in taste [9]. *D. zingiberensis* was firstly recorded in *Treatise on Cold Pathogenic Diseases*, a famous Traditional Chinese Medicine book. However, in this first report the medical application was listed in *Compendium of Materia Medica* medical monograph in Page 393. It was described to be useful for the treatment of abdominal distention, deadly cold hand and foot, inappetence, anorectic dysentery, frequent urination, deficiency of spleen and stomach, inhibiting initial swelling and etc. Generally, the rhizomes of *D. zingiberensis* are considered as part of TCM and have been widely used in China for many years. Several phytochemical investigations have revealed the variety of constituents comprising the matrix of extracted samples of *D. zingiberensis* and total steroid saponins (TSSN) mainly presenting in spirostan and furostan forms makes the largest portion of the constituents. Extracts of TSSN from this plant have demonstrated the signals that TSSN can be the potential bioactive ingredients for the treatment of various diseases, for example

cough, anthrax, rheumatthritis, tumefaction, sprain, cerebrovascular as well as cardiac diseases [10–12].

Particularly, *Dun-Ye-Guan-Xin-Ning* Capsule, a famous Chinese patent medicine extracted from the rhizome of *D. zingiberensis*, is reputed for its promising efficacy against cardiovascular diseases probably through the underlying mechanisms of increasing coronary blood flow, improving peripheral circulation as well as depressing platelet aggregation [13]. The acidic hydrolyzates of TSSN from *D. zingiberensis* are broadly utilized as raw materials to extract diosgenin after recrystallization by petroleum ether [14]. Diosgenin is an indispensable precursor to synthesize steroidal hormones like cortical hormone, sex hormone and progesterone in pharmaceutical industry [15]. Irrespective of its widespread use, extensive toxicological evaluation on TSSN from *D. zingiberensis* has never been done [16].

It is generally known that the toxicological evaluation is a crucial constituent part during drug development procedure. Therefore, the aim of the current study is to investigate the potential toxicity and safety profile of TSSN extracted from *D. zingiberensis* *in vivo* after making extensive efforts. A detailed experimental scheme was designed and the evaluation was performed through sub-acute and chronic oral administration of TSSN after a single dose and 3-month repeated doses on dogs, respectively. We hope that this finding may provide a preliminary scientific data on the toxicity profile of TSSN for pre-clinical trials and further scientific investigations for therapeutic applications.

2. Materials and methods

2.1. Plant materials and preparation of TSSN extracts from *D. zingiberensis*

D. zingiberensis, uniquely growing in China, are chiefly distributed in the mountainous places from the upper reaches of Hanjiang Basin of Wudang Mountain in Hubei province to the northern foot of the Qinling Mountains in Shaanxi province (Geographic Coordinates: 98°53′~121°50′ E, 23°42′~32°10′ N). This plant often grows in mountainous region, evergreen broad-leaved forest, mingled forest and river valley with an altitude of 100~1500 m. The rhizomes of this plant are often harvested from October to November.

Dried rhizomes of the cultivated *D. zingiberensis* that are harvested in November of 2016 were obtained from Yangtze River Pharmaceutical Industry Co., Ltd (Jiangsu, China). They were authenticated by Professor Wenji Sun of Northwest University (Shaanxi, China). A specimen voucher (No. Drsr009) has been deposited in the Biology and Medicine Key Laboratory of Shaanxi province of the same university. The TSSN extracts were prepared according to the description in our previous report [17].

2.2. Isolation and purification of monomers of TSSN via preparative HPLC

Partitions with different polarity were obtained after repetitive column chromatography and the separation process was consistent with the same processes used on other studies [17]. Firstly, aiming to obtain the composition of mobile phase on analytical HPLC, each partition was performed on a Waters 2695 serious liquid chromatograph system (Waters, Milford, MA, USA) containing a quaternary pump, an on-line vacuum degasser, an auto sampler and

a column temperature controller. The sample was separated on Welchrom C18 (250 mm×4.6 mm, 5 µm) at ambient temperature. The elution mobile phase was acetonitrile-water system under the flow rate of 1 mL/min. The evaporative laser scatter detector (ELSD, Alltech 2000ES) was chosen to monitor the effluent online with the following parameters: The drift tube temperature was 104 °C and the gas flow rate was 2.8 L/min. Secondly, the large-scale preparation of target ingredients was carried out on the semi-preparative HPLC called Alltech equipment system (Alltech CO. Ltd, California, USA) with a high pressure binary 627 HPLC pump and a 2 mL sample loop. The sample separation was conducted on semi-preparative column Econosil C18 (250 mm×22 mm, 10 µm) with acetonitrile-water system as the mobile phase in an isocratic elution mode. The amount of sample to be loaded was determined based on the resolution between target compound and the neighbouring peaks. The fraction was collected manually in line with the chromatogram and then was concentrated under reduced pressure to obtain residues. The structure was elaborated on Thermo LTQ-XL MS and Bruker Advance 500 NMR apparatuses.

2.3. Experimental animals and housing condition

The dogs (weighing 9~14 Kg) of both sexes purchased from the Experimental Animal Centre of Shaanxi Province (Shaanxi, China) were used for the sub-acute and chronic toxicity studies. The dogs were raised in an open feeding farm with a natural light. These animals were supplied with clean drinking water and standard pellet diet *ad libitum*. They were acclimatized the housing conditions at least for 7 days prior to the initiation of the experiment. All the animal experiments were strictly conducted in compliance with the Care and Use of Laboratory Animals based on the guidelines of NIH (pub. No. 85-23, Revised 1985). The experimental protocol was approved by the institutional animal care and use committee of Xi'an Jiaotong University. Extensive efforts were made to minimize suffering from pain and the number of animals being used.

2.4. Group assignment and experimental design

2.4.1. Sub-acute toxicity study of TSSN—Sixteen dogs were assigned into control and treatment groups each containing 4 male and 4 female animals. They were without food for 12 h prior to dosing. The TSSN extracts (1 g TSSN extract=25 g dried rhizomes) were dissolved in distilled water and administrated orally to the dogs in the treatment groups at a dose of 3 g/Kg (1.6 g/Kg human equivalent dose) by gavage according to their body weight. This treatment was repeated three times with a 3-h interval daily and maintained for 14 days, during which the control group orally received equal volume of distilled water. The dogs were weighed before and after the TSSN treatment. The medial lethal dose (LD₅₀) was calculated according to the previous method [18]. The experiment scheme was designed according to the technical guidelines of sub-acute toxicity for traditional Chinese medicine and natural drugs (No. [Z]GPT2-1) [19].

2.4.2. Chronic toxicity study of TSSN—Thirty-two dogs were randomly divided into control and three treatment groups of 8 animals each (4 males and 4 females). Dogs in treatment groups were orally administered with graduated doses of 50, 250 and 500 mg/Kg of TSSN in distilled water for a 3-month period, respectively, which amounted to 26.74, 133.69 and 267.38 mg/Kg human equivalent dose. Comparatively, the solvent medium was

used as vehicle in control group. The gradient dosages were chosen in accordance with LD₅₀ value obtained in sub-acute toxicity study and the dogs were kept alive. The body weight of each dog was recorded once a week throughout the experiment using an electronic balance. 8 dogs in each group with equivalent sex were sacrificed for further analysis at the end of the 3rd month, while the other remaining animals in each group were continuously fed to perform recovery test for two weeks and sacrificed at the end of the research. The experimental scheme was designed according to the technical guidelines of long term toxicity for traditional Chinese medicine and natural drugs (No. [Z]GPT3-1) [20].

2.5. Observations of general behaviors and toxic signs

The behavior, symptoms, toxic manifestations, latency of death and mortality were observed continually for 1 h after treatment, then intermittently for 4 h and over a 24-h period thereafter. This observation surveillance was conducted once a day and sustained for 14 consecutive days in the sub-acute toxicity study and 12 weeks following a 2-week recovery assay in chronic toxicity study. If any adverse effects for instance hypoactivity, anorexia, tremors, convulsions, salivation, diarrhea, lethargy, syncope and coma came up after dose administration, these signs were intermediately observed and recorded.

2.6. Preparation of blood sample and tissues

The dogs were left without food to fast for 12 hours but with free access to water before sacrificed. These animals were anesthetized with pentobarbital sodium solution and the blood samples were collected into two plastic tubes from the femoral vein. Blood in a tube containing ethylene diamine tetraacetate anticoagulant was immediately processed for the analysis of hematological parameters. Conversely, blood in another tube without any additive was standing at ambient temperature by roughly 30 min and then concentrated at 3500 rpm for 10 min. The supernatants were collected and the obtained serum was maintained at -20 °C for subsequent biochemical analysis.

After the blood collection, the animals were sacrificed to isolate and study structural and biochemical changes in the following organs: heart, liver, spleen, lungs, kidneys, brain, thymus, thyroid gland, pituitary gland, adrenal gland, prostate gland, uterus, ovary and testis. The residual liquid on the surface of these tissues was sucked to dryness and immediately weighed. The intact organs were used for necropsy and a small section of these tissues containing heart, liver, spleen, lungs, kidneys, brain, thymus, pancreas, thyroid gland, pituitary gland and adrenal gland were dissected and immobilized in 10% paraformaldehyde solution for histopathological examinations. The urine was directly collected from bladder and the urinalysis was conducted after pre-treatment.

2.7. Histopathological analysis

A comprehensive necropsy of the organs and tissues was macroscopically examined and the criteria of gross lesions including the shape, size, color, position, hardness of the outer membranes was utilized for necropsies. The relative organ weight was calculated as the organ or tissue weight divided by the body weight expressed in mg/g or g/g percent. Histopathological examination was operated on the control and high-dose treatment groups. The fixative tissues were made to be in a series of routine processing of paraffin embedding,

ascending grades of ethanol washing, xylene clearing and sectioning with a microtome and finally stained with hematoxylin and eosin (H&E). The morphological alterations of each section were macroscopically observed (Olympus, BH-2, Japan) and photomicrographs of lesions were recorded for further histopathological analysis.

2.8. Hematological and serum biochemical determination

The hematological analysis was carried out on a full BC-2000 automatic hematology analyzer (Mindray Co. Ltd, USA). The determined parameters were as follows: red blood cell count (RBC), hemoglobin concentration (HGB), white blood cell count (WBC), white blood cell classification, platelet (PLT) and blood clotting time (BCT). Meanwhile, the serum biochemical analysis was assayed using an XD-8IIc semi-automatic biochemical analyzer (Xunda Medical Instrument Co., LTD, Shanghai, China). These biochemical indicators were examined and listed as: alanine aminotransferase (ALT), aspartic acid transaminase (AST), alkaline phosphatase (ALP), total cholesterol (TCHO), total bilirubin (TBIL), total protein (TP), albumin (ALB), creatinine (CRE), blood urea nitrogen (BUN) and glucose (GLU). This evaluation was conducted using the kits purchased from BioSino Bio-technology and Science Inc. (Beijing, China) and according to the corresponding manufacturer's instructions.

2.9. Routine urinalysis

The following parameters in urine including glucose (GLU), bilirubin (BIL), ketone body (KET), urine protein (PRO), urobilinogen (URO), urine nitrite (UNIT), occult blood (UBLO) and pH were measured using kits purchased from Nanjing Jiancheng Biotechnology Co., Ltd (Nanjing, China).

2.10. Electrocardiographic examination

The dogs were fixed in the supine position after anesthetized with pentobarbital sodium solution at the end of the experiment. Their electrocardiograms were determined and recorded by Cardiofix apparatus according to the parameters of 1 mv/cm standard voltage, standard II lead and 25 mm/s speed.

2.11. Statistical analysis

The final results were expressed as mean value $\pm$ standard deviation (SD) and data processing was performed using SPSS version 23.0 (SPSS, Chicago, USA). Homogeneity of variance was determined by Levene's test. When the result is not significantly different, the values of the control and treatment groups were subjected to one-way analysis of variance (ANOVA), followed by Duncan's test. Since the variance obtained was not homogenous on our second analysis, non-parametric ANOVA test was applied, followed by Kruskal Wallis test. A probability level of less than 0.05 ($P < 0.05$) was considered as significantly different.

3. Results

3.1. Structure identification of steroid saponins

Twenty-five single constituents were eventually obtained from TSSN extracts of *D. zingiberensis* after an exhaustive chromatographic separation with the additional use of D101 macroporous adsorption resin, normal silicon gel and preparative HPLC, among which 8 compounds were initially isolated from this plant. It is a common sense that steroid saponins are mainly composed with aglycones and sugar moieties via glucosidic bond linkage. The sugar composition through a comparison of chromatographic behaviors with the reference standard sugars on TLC was elaborated after acidolysis of steroid saponins. The outcomes illuminated glucose and rhamnose were the principal constituent sugar groups. The monomer steroid saponin was determined as furostan type if the color development was pink with E reagent, and spirostan type if no color appeared. The structures of aglycones and the sugar moieties of these 25 ingredients were summarized in Fig. 1 and Table 1.

3.2. Sub-acute oral toxicity study

In the sub-acute toxicity study, the dogs in the treated groups revealed a normal activity following oral administration of TSSN extracts at a single dose level up to 3 g/Kg throughout a 14-day period. No abnormal behavior changes or treatment associated with toxic signs such as agitation, uneasiness, convulsions, piloerection, lacrimation, salivation, convex eyes, diarrhea, writhing reaction, polypnea and cyanosis of lip as well as tail appeared in both gender of the dogs. Furthermore, as compared with the initiation average body weight at 10.13 ± 1.56 Kg and 11.15 ± 1.24 Kg of male and female, respectively, body weight gain at 11.39 ± 1.22 Kg and 12.04 ± 1.57 Kg was observed at the end of the experiment. The necropsy examination demonstrated that these internal organs afore-obtained in the section “2.6” from each animal presented a normal morphology in size and color. Since no mortality phenomenon was emerged in both genders of the control and treatment groups of dogs throughout the sub-acute toxicity study, the LD₅₀ of TSSN on dogs couldn't be calculated and this value was probably greater than 3 g/Kg.

3.3. Chronic toxicity study

3.3.1. General behaviors, body weight evaluation and relative organ weight—

Similar to the sub-acute toxicity study, neither observable toxic signs nor death case were recorded in both gender dogs of the treatment groups through the consecutive 3-month period and during the recovery test after administration of TSSN extracts at either dosage of 50, 250 and 500 mg/Kg. Lack of noticeable difference in fur, activity, general behaviors, food and drink intake, urine and feces was the main finding in addition to a rhythmic breath, sensitive reaction and natural muscular tension compared with the control dogs. The body weight variations of these dogs were documented and vividly presented in Fig. 2. No significant treatment-related changes were observed as compared to the vehicle treated dogs. However, an increase in body weights compared with the one at the begging of the experiment within each group was presented, indirectly suggesting that TSSN at the determined dosages couldn't have an impact on appetites of these animals. In terms of relative organ weights, as shown in Table 2, there was no significant difference caused by

TSSN-treatment in heart, liver, spleen, lungs and kidneys throughout the study period in comparison with control group.

3.3.2. Macroscopic examination and histopathological analysis—The necropsy results showed that the organs listed in Table 2 displayed normal shapes, color and lustre as compared with control dogs at the end of TSSN-treatment and during the recovery experiments after macroscopic observations. These findings were simultaneously verified after histopathological investigations of the heart, liver, pancreas, spleen, lungs, kidneys, brain, thyroid gland, adrenal gland, thymus gland, and pituitary gland. From Figs. 3–4, it is possible to draw a conclusion that absence of any gross severe pathological changes like hemorrhagic spot, cellular shrinking and cellular contents exudation was observed in H&E stained sections. These tissues presented a normal morphology with an unambiguous boundary, particle and intercellular space in the dogs subjected to TSSN extracts when compared with control group.

3.3.3. Effect of TSSN on haematological and biochemical parameters—The haematological and biochemical data in the control and treatment groups were summarized in Tables 3–4, respectively, at the end of 3-month treatment and recovery period. It was apparent from these tables that the determined haematological parameters including RBC, HGB, WBC, WBC classification, PLT and BCT were obviously altered in both sexual dogs as compared to the normal group. However, their values remained in the normal range throughout chronic treatment period. Similarly, no adverse variations of biochemical profiles in ALT, AST, ALP, TCHO, TBIL, TP, ALB, CRE, BUN and GLU were observed in male and female animals after repeated oral administration of TSSN extracts at daily dosage of 50, 250 and 500 mg/Kg throughout experimental duration.

3.3.4. Routine urine examination—The urinary findings were integrated in Table 5 after finishing the chronic toxicity evaluation. It was deduced from this table that no detectable concentration of GLU, BIL, KET, PRO, URO, UNIT and UBLO in urine was found in each animal of both the control and treatment groups. In addition, the pH numerical value was detected in the range of 6~7 and the urine was neutral condition. These afore-stated results demonstrated that kidney was not injured when the dogs were administered with TSSN extracts at three different dose levels throughout the experiment. Certainly, this evidence believed to be consistent with the histopathological analysis of the kidney.

3.3.5. Electrocardiographic analysis—The electrocardiograms were recorded at the end of 3-month treatment and recovery test periods after the administration of TSSN extracts to dogs and the representative images of control and 500 mg/Kg dose were presented in Fig. 5. The overall outline of high-dose TSSN administration was almost the same with the control dogs along with characteristic features of each containing P, Q, R, S and T waves. There were no significant changes in the range of QRS and ST waves. This phenomenon was discovered after the recovery test and these outcomes inferred as the heart function properly in the chronic study of TSSN. The finding of the histopathologic examination of the heart also attests this outcome and allows us to draw the conclusion that TSSN was non-toxic.

4. Discussion

Many patients have now paid their attention to TCM, a characteristic medicine system that has been practiced for thousands of years, for treatment of various ailments due to moderate efficacy and less side effects. However, the fact that it has some side effects, adverse reactions or toxicities, can't be denied. Therefore, together with its promising therapeutic effects, toxicological investigation of TCM on experimental animals is indispensable to provide for an appropriate dosage selection for its clinical use. In recent years, *D. zingiberensis*, an ethno pharmacological and traditional TCM, has demonstrated to exert some newly emerging bioactivities as afore-mentioned in the “**introduction section**” and triggered our interest to explore its chemical composition for pharmacological and clinical applications in depth with extensive efforts [10–12]. The essential TSSN extracts originated from *D. zingiberensis* have been elaborated as the bioactive compounds that are attributable to the observable effects according to our previous chemical and pharmacological investigations which provided the concentration of some biomarker constituents [17, 21, 22]. In spite of the increasing bioactivity data, the detailed available information regarding the potential toxicity *in vivo* of TSSN from this plant is relatively limited to an anterior report about cursory evaluation on mice and rats [16]. With the purpose of updating and enriching the pre-existing toxicological data, a comprehensive assay of TSSN was carried out separately via a sub-acute and a chronic toxicity study on dogs.

Sub-acute toxicity study was an initial step to perform the systematic toxicity evaluation of drugs and offer clues for subsequent chronic toxicity studies following the trails on a wide range of dosages. In this sub-acute toxicity study, no treatment-related general behavior changes and toxic manifestations appeared when the dogs were subjected to TSSN extracts at the dose of 3 g/Kg by gavage for 2-week duration compared with the control animals. Surprisingly, all animals survived until the scheduled euthanasia, suggesting that the extracts were well tolerated by the experimental dogs and even at the doses of LD₅₀ greater than 3 g/Kg. According to the toxic hazard classification and the report on another study [18], the absence of toxicity could be considered as the pharmacologic property of the plants extracts or the plant products when the LD₅₀ value exceeded 2~3 g/Kg. They were non-toxic provided that there were no bad clinical signs and no recorded deaths at doses of below 5 g/Kg [23]. Above all, it seemed reasonable to presume that TSSN extracts from *D. zingiberensis* revealed no significant toxicity. Our research group was exerting no efforts to develop TSSN extracts into a new drug which was utilized to treat cardiovascular disease with satisfactory effectiveness. This research has applied a patent (ZL03108036.7.3, ZL200910218678.6) and the daily presupposed dose was determined to be maximum 600 mg. Given the fact that an average body weight of an adult was 60 Kg [24], the dose was 10 mg/Kg. When the lack of toxicity of this extracts validated, the selected dose of 3 g/Kg in sub-acute study becomes 300 times of the clinical dosage. A series of doses of total steroidal saponins (TSSN) extracts were selected to determine the chronic toxicity on dogs in preliminary experiments. When the dose was 500 mg/Kg, no adverse effect, for example agitation, uneasiness, convulsions, piloerection, lacrimation, salivation, convex eyes, etc., induced by TSSN was observable on dogs throughout a 90-day treatment following a 14-day recovery period. In addition, no mortality was recorded in this detected dose. According to

the definition of no observed adverse effect level (NOAEL), 500 mg/Kg was considered as the highest dose that couldn't induce adverse effects on dogs in this experiment [25]. The human equivalent dose (HED) and maximum recommending starting dose (MRSD) was calculated according to the following formulae: $HED = \text{animal NOAEL} / \text{conversion factor}$, $MRSD = HED / \text{safety factor } 10$. The conversion factor of dog to human was 1.87, therefore, HED and MRSD was calculated as 267 mg/Kg and 26.7 mg/Kg, respectively.

The chronic study for 3-month period and recovery test for 2-week stage, together with sub-acute toxicity evaluation, has been applied as the fundamental approach for the comprehensive study of the safety profile, determination of the toxic effects, identification of target organs prone to damage and prediction of latent toxic response [6]. The employed gradient doses in this study were finally designed as 50, 250 and 500 mg/Kg based on the LD_{50} calculated in the sub-acute toxicity research which is equivalent to one-sixtieth, one-twelfth and one-sixth of LD_{50} , respectively. Alterations in general behavior, body weight as well as organ weight and analysis of biochemical and hematologic parameters are the diagnostic indicators to evaluate the adverse effects of drugs and chemicals and provide useful references whenever the experimental data are translated from animal studies to clinical applications [26, 27]. Therefore, being selected to determine the potential toxicity of TSSN extracts in our study compared with the control dogs, the experimental dogs remained unaffected due to treatment, when chronically exposed to TSSN extracts at the aforementioned three dose levels for 3-months and 2-week recovery test. Whenever the body is subjected to endogenous and exogenous harmful stimuli, the hematopoietic system and blood profiles are the most sensitive targets to primarily make urgent response which reacts to the physiological and pathological status of our homeostasis [28]. Plenty of primary data implied that TSSN extracts from *D. zingiberensis* probably exhibited no toxicological effects on the hemopoietic system. Besides, there were no unusual variations on some indices with respect to the normal functions of the heart, liver and kidneys after treatment as compared to the control dogs. The findings suggested that the accumulating phenotype or even the metabolites of the plant extracts couldn't impose any damages to the organs after the repeated oral administration for the chronic toxicity study period. This discovery was further supported by the histopathological examination which showed normal architecture without morphological disturbances. Thus, it was reasonable to draw the conclusion that chronic administration of TSSN to dogs at the determined dosages was safe enough and produced no detrimental effects to the body function which complements with the outcomes obtained in the sub-acute toxicity study.

As the multifarious bioactivities of a plant or its derived products are attributed to the chemical constituents existing in a complex matrix, so does the toxicity of a plant. Our separation and identification research work via preparative HPLC and HPLC-MS/MS, respectively, demonstrated that the steroid saponins were classified into spirostan and furostan types which were in line with a previous report [29]. This phytochemical composition could provide instructional information for toxicology research on TSSN extract from *D. zingiberensis*. The steroid saponins were possibly decomposed into their corresponding constituent aglycones and sugar moieties via acidolysis under acid environment of the stomach. The secondary substances were further metabolized into other small molecule through methylation, hydroxylation, etc. under assistance of these enzymes

and bacteria that abundantly inhabit in the digestive system such as the small intestine. These metabolites themselves were found to be non-toxic indicating the lack of toxicity of TSSN extracts after oral administration when the less polar constituents reached the target organs after passing through the cell membranes. Furthermore, steroid saponins were potential lytic agents and could cause lysis of red blood cells *in vitro* [30]. These toxic phenomena of hemolytic anemia and relevant alterations of hematological parameters were not observed in this investigation. Steroid saponins were a type of intensively polar compounds led by the constituent sugar groups and they except for their metabolites could pass through the membrane. However, this ability was weak and trace of them was determined in blood, therefore, our previous reports have demonstrated that they displayed a low bioavailability after oral administration of TSSN from *D. zingiberensis* [13, 31, 32]. We hypothesized another possibility that the concentration of steroid saponins in blood, tissue and organs couldn't reach threshold values to cause the claimed toxic responses due to their low bioavailability pharmacokinetic features indicating the lack of toxicity of TSSN.

A similar toxicity evaluation of steroid saponins from *D. zingiberensis* was performed on mice and rats in sub-acute and chronic toxicity study, respectively [16]. Nonetheless, two diverse conclusions were finally drawn from these two studies. Steroid saponins from the same plant have previously been classified as toxic with LD₅₀ at an oral dose of 3653 mg/Kg, conversely, the TSSN extracts revealed non-toxic effects at up to the dose of 3000 mg/Kg. After comparing the two experimental details and consulting the corresponding references, we considered three reasons could explain the different findings and they were particularly elaborated as follows. First of all, the matrix chemical constitution of steroid saponins administrated with animals was distinct although they were both extracted from *D. zingiberensis*. Based on the previous description, the plant preparation was directly obtained when the raw materials were soaked for 12 h with ethanol solution and then extracted with hot ethanol under reflux thrice. While, the TSSN preparation was obtained in line with the following procedures including extraction with 70% ethanol solution thrice each with 2 h, separation on D101 macro reticular resin and elution with 60% ethanol, extraction with n-butanol and subsequent concentration. It could be deduced that the chemical composition of former extracts was more complex than the latter one in terms of separation procedures, i.e. the concentration of pure steroid saponins in TSSN extracts was higher. Likely, other compounds finally caused the treatment-related toxicity other than steroid saponins in previous reports. Secondly, the experimental animals were different in which the toxic response observed. Mice and rats were utilized in an early study while dogs were used in the present study. Diverse animals probably had different susceptibilities to the toxicity of the same drugs and to be inflicted by the subsequent toxic reactions. Eventually, the administrated dosages were different and the dose-dependent relationship accounted for the occurrence of the toxicity. Certainly, the above explanations were hypothesis and experimental schemes with details should be carried out to verify the rationale behind.

5. Conclusions

Collectively, lack of mortality, adverse changes in general behaviours, body weight as well as relative organ weight and alterations in haematological, biochemical and histopathological analysis didn't appear and remained as the variables in the normal range

when the dogs were exposed to TSSN extracts from *D. zingiberensis* at dose of 3000 mg/Kg in sub-acute toxicity study for 14 days and in chronic toxicity study at three doses of 50, 250 and 500 mg/Kg for 90 days with subsequent recovery test for 14 days. Based on the study result, it was possible to draw a faithful conclusion that this herbal preparation can be considered as non-toxic after the thorough rough toxicological evaluations and the dogs were able to tolerate the extracts at dose up to 500 mg/Kg with LD₅₀ greater than 3000 mg/Kg. The overall outcomes could provide useful preliminary scientific information on the medical application whenever TSSN extracts are translated from experimental stage to clinical use.

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References

- Li FJ, He XL, Niu WY, Feng YN, Bian JQ, Xiao HH. Acute and sub-chronic toxicity study of the ethanol extract from leaves of *Aralia elata* on rats. *J Ethnopharmacol.* 2015; 175:499–508. [PubMed: 26456344]
- Williamson, EM., Okpako, DT., Evans, FJ. Selection, preparation and pharmacological evaluation of plant materials. John Wiley and Sons; Chichester, England: 1996.
- Zhu M, Lew KT, Leung P. Protective effects of plants formula in ethanol-induced gastric lesions in rats. *Pytother Res.* 2002; 16:276–280.
- Saad B, Azaizah H, Abu-Hijleh G, Said O. Safety of traditional Arab herbal medicine. *Evid-based Complement Altern Med.* 2006; 3:433–439.
- Ezeji MI, Anaga AO, Asuzu IU. Acute and sub-chronic toxicity profile of methanol leaf extract of *Gouania longipetala* in rats. *J Ethnopharmacol.* 2014; 151:1155–1164. [PubMed: 24384377]
- Xu KK, Lin Y, Zhang RT, Lan M, Chen CY, Li SH, Zuo CY, Chen C, Zhang TN, Yan ZY. Evaluation of safety of iridoids rich fraction from *Valeriana jatamansi* Jones: Acute and sub-chronic toxicity study in mice and rats. *J Ethnopharmacol.* 2015; 172:386–394. [PubMed: 26164073]
- Nghonjuyi NW, Tiambo CK, Taiwe GS, Toukala JP, Lisita F, Julianio RS, Kimbi HK. Acute and sub-chronic toxicity studies of three plants used in Cameroonian ethnoveterinary medicine: *Aloe vera* (L.) Burm. f (Xanthorrhoeaceae) leaves, *Carica papaya* L (Caricaceae) seeds or leaves, and *Mimosa pudica* L (Fabaceae) leaves in Kabir chicks. *J Ethnopharmacol.* 2016; 178:40–49. [PubMed: 26657577]
- Yang R, Tang S, Pan F, Zhao A, Pang Z. Advances in study of *Dioscorea zingiberensis*. *Chinese Wild Plant Resources.* 2007; 26:1–5.
- Liu L, Dong YS, Xiu ZL. Three-liquid-phase extraction of diosgenin and steroidal saponins from fermentation of *Dioscorea zingiberensis* C.H. Wright. *Process Biochem.* 2010; 45:752–756.
- Zhang XX, Ito Y, Liang JR, Liu JL, He J, Sun WJ. Therapeutic effects of total steroid saponin extracts from the rhizome of *Dioscorea zingiberensis* C.H. Wright in Freund's complete adjuvant induced arthritis in rats. *Int Immunopharmacol.* 2014; 23:407–416.
- Zhang XX, Chen L, Dang X, Liu JL, Ito Y, Sun WJ. Neuroprotective effects of total steroid saponins on cerebral ischemia injuries in an animal model of focal ischemia/reperfusion. *Planta Med.* 2014; 80:637–644. [PubMed: 24963614]
- Zhang XX, Chen L, Liu JL, Ito Y, He J, Sun WJ. Neuroprotection of total steroid saponins from *Dioscorea zingiberensis* against transient focal cerebral ischemia-reperfusion injury in rats via

anti-inflammatory and anti-apoptotic effects. *Planta Med.* 2014; 80:1597–1604. [PubMed: 25340467]

13. Zhang XX, Li J, Ito Y, Sun WJ. Simultaneous quantification of five steroid saponins from *Dioscorea zingiberensis* C.H. Wright in rat plasma by HPLC-MS/MS and its application to the pharmacokinetic studies. *Steroids.* 2015; 93:16–24. [PubMed: 25201262]
14. Gong GH, Qin Y, Huang W. Anti-thrombosis effect of diosgenin extract from *Dioscorea zingiberensis* C.H. Wright *in vitro* and *in vivo*. *Phytomedicine.* 2011; 18:458–463. [PubMed: 21036572]
15. Du D, Zhang R, Xing ZH, Liang YY, Li SF, Jin T, Xia Q, Long D, Xin G, Wang GZ, Huang W. 9, 10-Dihydrophenanthrene derivatives and one 1, 4-anthraquinone firstly isolated from *Dioscorea zingiberensis* C.H. Wright and their biological activities. *Fitoterapia.* 2016; 109:20–24. [PubMed: 26656408]
16. Qin Y, Wu XH, Huang W, Gong GH, Li D, He Y, Zhao YL. Acute toxicity and sub-chronic toxicity of steroidal saponins from *Dioscorea zingiberensis* C.H. Wright in rodents. *J Ethnopharmacol.* 2009; 126:543–550. [PubMed: 19735710]
17. Zhang XX, Ito Y, Liang JR, Su Q, Zhang YM, Liu JL, Sun WJ. Preparative isolation and purification of five steroid saponins from *Dioscorea zingiberensis* C.H. Wright by counter-current chromatography coupled with evaporative light scattering detector. *J Pharmaceut Biomed.* 2013; 84:117–123.
18. Horn HJ. Simplified LD₅₀ (or ED₅₀) calculations. *Biometrics.* 1956; 12:311–322.
19. No CFDA. [Z]GPT21: technical guidelines of acute toxicity for Traditional Chinese Medicine and natural drugs. CFDA Guidelines for the Evaluation of Drug. 2005b:1–8.
20. No CFDA. [Z]GPT31: technical guidelines of long term toxicity for Traditional Chinese Medicine and natural drugs. CFDA Guidelines for the Evaluation of Drug. 2005c:1–16.
21. Zhang XX, Liang JR, Liu JL, Zhao Y, Gao J, Sun WJ, Ito Y. Quality control and identification of steroid saponins from *Dioscorea zingiberensis* C.H. Wright by fingerprint with HPLC-ELSD and HPLC-ESI-Quadrupole/Time-of-flight tandem mass spectrometry. *J Pharmaceut Biomed.* 2014; 91:46–59.
22. Zhang XX, Liang JR, Su Q, Xie RM, Sun WJ. Simultaneous content determination of five saponins in the rhizome of *Dioscorea zingiberensis* C.H. Wright by HPLC-ELSD. *Chin J Pharm Anal.* 2013; 33(7):1235–1238.
23. Brock WJ, Trochimowicz HJ, Millischer RJ, Farr C, Kawano T, Rusch GM. Acute and sub-chronic toxicity of 1, 1-dichloro-1-fluoroethane (HCFC-141b). *Food Chem Toxicol.* 1995; 33:483–490. [PubMed: 7797175]
24. Van Miert, ASJPAM. The use in animals of drugs licensed for human use only. In: Van Miert, ASJPAM, Bogaert, MG., Debackere, M., editors. *Comparative Veterinary Pharmacology, Toxicology and Therapy.* MTP Press; Boston: 1986. p. 489-500.
25. Bae JW, Kim DH, Lee WW, Kim HY, Son CG. Characterizing the human equivalent dose of herbal medicines in animal toxicity studies. *J Ethnopharmacol.* 2015; 162:1–6. [PubMed: 25543017]
26. Tofovic SP, Jackson EK. Effects of long-term caffeine consumption on renal function in spontaneously hypertensive heart failure prone rats. *J Cardiovasc Pharmacol.* 1999; 33:360–366. [PubMed: 10069669]
27. Carrillo JC, Adenuga MD, McKee RH. The sub-chronic toxicity of regular White Spirit in rats. *Regul Toxicol Pharmacol.* 2014; 70:222–230. [PubMed: 25013991]
28. Mukinda JT, Eagles FK. Acute and sub-chronic oral toxicity profile of the aqueous extract of *Pohy gala fruticosain* female mice and rats. *J Ethnopharmacol.* 2010; 128:236–240. [PubMed: 20079821]
29. Zhu JB, Guo XJ, Fu SP, Zhang XL, Liang XM. Characterization of steroidal saponins in crude extracts from *Dioscorea zingiberensis* C.H. Wright by ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *J Pharmaceut Biomed.* 2010; 53:462–474.
30. Baumann E, Stoya G, Volkner A, Richter W, Lemke C, Linss W. Hemolysis of human erythrocytes with saponin affects the membrane structure. *Acta Histochem.* 2000; 102:21–35. [PubMed: 10726162]

31. Zhang XX, Xue XJ, Zhao J, Guo ZJ, Ito Y, Sun WJ. Quantitative determination of gracillin by HPLC-MS/MS after oral administration and its application to a pharmacokinetic study. *Steroids*. 2016; 113:78–86. [PubMed: 27394958]
32. Zhang XX, Guo ZJ, Li J, Ito Y, Sun WJ. A new quantitation method of protodioscin by HPLC-ESI-MS/MS in rat plasma and its application to the pharmacokinetic study. *Steroids*. 2016; 106:62–69. [PubMed: 26703445]

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Total steroid saponins from *Dioscorea zingiberensis* C.H. Wright reveal bioactivities.

The sub-acute and chronic toxicity studies of TSSN were investigated on dog animals.

The LD₅₀ was calculated greater than 3000 mg/Kg.

No toxic appearance was observable after examining the corresponding parameters.

Figure 1.
The chemical structure of aglycones consisting of steroid saponins that isolated via conventional columns and preparative HPLC.

Figure 2.

Mean body weight changes of male and female dogs exposed to TSSN extracts at three doses of 50, 250 and 500 mg/Kg in the chronic toxicity study for 12 weeks and 2 weeks recovery test.

Figure 3.

Typical microphotographs of organ histopathological examination of dogs after administrated with TSSN for 3-month period in chronic toxicity study. The left column represented the organs of normal dogs, while the right of the high dose of 500 mg/Kg. Original magnification 40 x

(a) Heart; (b) Liver; (c) Spleens; (d) Lungs; (e) Kidney; (d) Brain

Figure 4. Typical microphotographs of organ histopathological examination of dogs after administrated with TSSN for 3-month period in chronic toxicity study. The left column represented the organs of normal dogs, while the right of the high dose of 500 mg/Kg. Original magnification 40 x
(a) Thyroid gland; (b) Adrenal gland; (c) Thymus gland; (d) Pancreatic gland; (e) Pituitary gland

Figure 5.

The representative electrocardiograms of dogs after chronic exposure to TSSN extracts.

(a) Normal group at the end of 3-month study; (b) 500 mg/Kg at the end of 3-month study;

(c) Normal group at the end of recovery test; (d) 500 mg/Kg at the end of recovery test

Table 1

The sugar moieties of isolated compounds from *Dioseorea zingiberensis* C.H. Wright and the corresponding aglycones that presented in Figure 1.

Compound NO.	R ₁	R ₂	Types of aglycone
1	Glc(1→3)-Glc(1→4)-Rha(1→2)-Glc	Glc	Type I
2	Rha(1→2)[Glc(1→4)]Glc	Glc	Type I
3	Rha(1→4)-Rha(1→4)[Rha(1→2)]-Glc	Glc	Type I
4	Rha(1→2)[Glc(1→3)]-Glc	Glc	Type II
5	Glc(1→4)[Rha(1→2)]-Glc	Glc	Type II
6	Rha(1→4)-Glc	Glc	Type II
7*	Rha(1→4)-Rha(1→4)[Rha(1→2)]-Glc	Glc	Type II
8*	Glc(1→6)-Glc(1→3)-Glc(1→4)[Rha(1→2)]-Glc	Glc	Type II
9	Rha(1→2)-Glc	–	Type III
10	Rha(1→4)[Rha(1→2)]-Glc	–	Type III
11	Glc(1→3)[Rha(1→2)]-Glc	–	Type III
12	Rha(1→3)[Glc(1→2)]-Glc	–	Type III
13	Rha(1→4)-Rha(1→4)[Rha(1→2)]-Glc	–	Type III
14	Glc(1→3)-Glc(1→4)[Rha(1→2)]-Glc	–	Type III
15*	Glc(1→4)Rha[(1→2)]-Glc	Glc	Type IV
16*	Rha(1→4)[Rha(1→2)]-Glc	Glc	Type IV
17*	Rha(1→2)-Glc	–	Type V
18*	Rha(1→4)[Rha(1→2)]-Glc	–	Type V
19	Glc(1→3)-Glc(1→2)[Rha(1→4)]-Glc	Glc	Type VI
20*	Glc(1→3)-Glc(1→4)[Rha(1→2)]-Glc	Glc	Type VII

Note: The asterisk in the upper right corner represents the compounds that firstly isolated from *Dioseorea zingiberensis* C.H. Wright

Table 2

The relative organ weight after treatment with total steroid saponins for 3-month period on dogs in chronic toxicity investigation.

Time Interval	Organs	Group (n=4)							
		Normal Control		50 mg/Kg		250 mg/Kg		500 mg/Kg	
		Male	Female	Male	Female	Male	Female	Male	Female
3 rd month	Heart (g/%)	0.83 ± 0.06	0.81 ± 0.12	0.80 ± 0.05	0.79 ± 0.17	0.82 ± 0.09	0.83 ± 0.21	0.81 ± 0.10	0.83 ± 0.14
	Liver (g/%)	2.82 ± 0.65	2.96 ± 0.49	3.08 ± 0.67	2.55 ± 0.45	2.94 ± 0.63	3.01 ± 0.51	2.89 ± 0.81	2.45 ± 0.24
	Spleen (g/%)	0.53 ± 0.15	0.47 ± 0.12	0.50 ± 0.08	0.69 ± 0.03	0.52 ± 0.13	0.48 ± 0.23	0.49 ± 0.09	0.56 ± 0.11
	Lungs (g/%)	1.39 ± 0.19	1.75 ± 0.28	1.11 ± 0.72	1.71 ± 0.25	1.22 ± 0.12	1.48 ± 0.17	1.28 ± 0.81	1.47 ± 0.24
	Kidneys (g/%)	0.38 ± 0.04	0.42 ± 0.19	0.33 ± 0.07	0.41 ± 0.11	0.37 ± 0.05	0.39 ± 0.11	0.35 ± 0.05	0.32 ± 0.13
	Brain (g/%)	0.57 ± 0.08	0.55 ± 0.05	0.53 ± 0.12	0.53 ± 0.23	0.59 ± 0.09	0.61 ± 0.24	0.58 ± 0.13	0.49 ± 0.18
	Thymus gland (mg/%)	61.87 ± 13.80	58.12 ± 12.74	52.98 ± 6.25	62.25 ± 3.29	63.55 ± 3.15	55.46 ± 4.65	54.40 ± 10.35	57.74 ± 4.62
	Thyroid gland (mg/%)	6.52 ± 0.80	6.44 ± 1.26	6.17 ± 1.20	5.94 ± 0.75	9.72 ± 0.98	5.53 ± 0.24	6.12 ± 1.34	5.83 ± 1.81
	Pituitary gland (mg/%)	0.67 ± 0.06	0.74 ± 0.03	0.65 ± 0.11	0.69 ± 0.25	0.61 ± 0.13	0.68 ± 0.24	0.65 ± 0.10	0.72 ± 0.25
	Adrenal gland (mg/%)	22.82 ± 2.53	21.91 ± 1.51	20.83 ± 2.00	19.47 ± 2.13	20.94 ± 1.95	26.75 ± 1.36	21.05 ± 3.62	20.67 ± 2.49
the last 2 weeks	Prostate gland (g/%)	0.10 ± 0.02	—	0.11 ± 0.03	—	0.09 ± 0.02	—	0.11 ± 0.03	—
	Uterus (g/%)	—	0.26 ± 0.04	—	0.24 ± 0.04	—	0.21 ± 0.03	—	0.23 ± 0.04
	Ovary (mg/%)	—	23.28 ± 4.23	—	22.75 ± 1.41	—	22.94 ± 4.22	—	22.01 ± 2.15
	Testis (g/%)	0.12 ± 0.02	—	0.11 ± 0.02	—	0.12 ± 0.03	—	0.10 ± 0.02	—
	Heart (g/%)	0.83 ± 0.01	0.78 ± 0.13	0.81 ± 0.10	0.87 ± 0.18	0.84 ± 0.03	0.79 ± 0.25	0.82 ± 0.04	0.87 ± 0.14
	Liver (g/%)	2.98 ± 0.34	2.94 ± 0.99	3.21 ± 0.75	2.18 ± 0.14	3.14 ± 0.11	3.14 ± 0.41	3.10 ± 0.51	3.21 ± 0.31
	Spleen (g/%)	0.56 ± 0.09	0.69 ± 0.81	0.61 ± 0.99	0.64 ± 0.39	0.59 ± 0.07	0.59 ± 0.14	0.55 ± 0.10	0.54 ± 0.37
	Lungs (g/%)	1.26 ± 0.23	1.27 ± 0.26	1.27 ± 0.41	1.34 ± 0.21	1.15 ± 0.07	1.52 ± 0.23	1.25 ± 0.30	1.34 ± 0.13
	Kidneys (g/%)	0.36 ± 0.09	0.29 ± 0.05	0.37 ± 0.10	0.41 ± 0.07	0.38 ± 0.21	0.39 ± 0.03	0.33 ± 0.01	0.32 ± 0.17
	Brain (g/%)	0.52 ± 0.06	0.65 ± 0.11	0.53 ± 0.13	0.66 ± 0.21	0.55 ± 0.04	0.57 ± 0.28	0.59 ± 0.01	0.58 ± 0.28
	Thymus gland (mg/%)	60.52 ± 2.69	64.21 ± 4.21	60.53 ± 4.94	59.47 ± 3.43	63.63 ± 7.65	56.44 ± 2.63	62.18 ± 7.28	64.95 ± 5.26
	Thyroid gland (mg/%)	6.32 ± 1.56	5.68 ± 1.35	6.37 ± 1.54	6.14 ± 1.81	6.42 ± 0.29	6.91 ± 2.31	6.73 ± 0.70	6.71 ± 2.51
	Pituitary gland (mg/%)	0.68 ± 0.06	0.79 ± 0.13	0.65 ± 0.10	0.58 ± 0.19	0.66 ± 0.06	0.62 ± 0.12	0.65 ± 0.04	0.61 ± 0.21
	Adrenal gland (mg/%)	22.60 ± 3.96	26.14 ± 3.89	23.05 ± 5.16	22.37 ± 2.61	21.30 ± 4.10	25.41 ± 1.37	23.60 ± 0.57	24.31 ± 3.27
	Prostate gland (g/%)	0.11 ± 0.00	—	0.12 ± 0.00	—	0.10 ± 0.00	—	0.09 ± 0.00	—
	Uterus (g/%)	—	0.25 ± 0.00	—	0.21 ± 0.00	—	0.22 ± 0.00	—	0.20 ± 0.00

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Time Interval	Organs	Group (n=4)					
		Normal Control		50 mg/Kg		250 mg/Kg	
		Male	Female	Male	Female	Male	Female
	Ovary (mg/%)	—	24.22 ± 0.00	—	22.13 ± 0.00	—	20.18 ± 0.00
	Testis (g/%)	0.12 ± 0.00	—	0.10 ± 0.00	—	0.09 ± 0.00	—
500 mg/Kg		0.11 ± 0.00		—		—	
		24.02 ± 0.00		—		—	

Evaluation some hematological parameters of dogs after oral administrated with total steroid saponins for 3-month period in chronic toxicity exploration.

Table 3

Treatment		Parameters (Mean $\pm$ SD, n=4)						
		WBC Classification (%)						
Gender	Dosage (mg/Kg)	RBC (x10 ¹² /L)	HGB (g/L)	WBC (x10 ⁹ /L)	Lymphocyte	Neutrophil	PLT (x10 ⁹ /L)	BCT (s)
Male: 3 rd month	Normal	6.43 $\pm$ 0.45	150.83 $\pm$ 9.50	4.00 $\pm$ 0.75	12.00 $\pm$ 1.22	71.20 $\pm$ 0.78	75.67 $\pm$ 3.49	10.50 $\pm$ 0.87
	50	5.98 $\pm$ 0.61	154.67 $\pm$ 7.18	4.40 $\pm$ 1.43	14.30 $\pm$ 0.79	69.70 $\pm$ 0.69	218.50 $\pm$ 3.29	10.00 $\pm$ 0.37
	250	6.37 $\pm$ 0.37	153.83 $\pm$ 3.50	4.63 $\pm$ 1.55	2.31 $\pm$ 0.64	4.30 $\pm$ 0.45	186.00 $\pm$ 2.99	9.83 $\pm$ 0.40
	500	5.93 $\pm$ 0.69	148.50 $\pm$ 12.55	4.13 $\pm$ 0.78	12.60 $\pm$ 0.42	70.80 $\pm$ 0.54	165.67 $\pm$ 4.72	11.50 $\pm$ 3.27
Male: the last 2 weeks	Normal	6.18 $\pm$ 1.05	147.00 $\pm$ 4.22	5.55 $\pm$ 0.21	10.51 $\pm$ 0.42	73.91 $\pm$ 4.46	138.50 $\pm$ 3.54	11.00 $\pm$ 1.44
	50	5.62 $\pm$ 1.05	146.50 $\pm$ 4.58	5.45 $\pm$ 0.21	11.62 $\pm$ 0.42	75.20 $\pm$ 4.46	191.50 $\pm$ 3.54	12.00 $\pm$ 1.44
	250	5.58 $\pm$ 1.23	144.50 $\pm$ 5.41	6.65 $\pm$ 5.40	14.85 $\pm$ 1.61	70.90 $\pm$ 6.95	174.50 $\pm$ 5.45	11.05 $\pm$ 2.50
	500	5.98 $\pm$ 1.56	150.00 $\pm$ 4.47	5.40 $\pm$ 0.57	16.05 $\pm$ 7.42	69.50 $\pm$ 5.22	154.00 $\pm$ 3.11	12.50 $\pm$ 0.71
Female: 3 rd month	Normal	5.97 $\pm$ 1.13	149.43 $\pm$ 5.87	5.01 $\pm$ 0.84	12.16 $\pm$ 0.85	63.02 $\pm$ 3.42	177.00 $\pm$ 6.07	10.44 $\pm$ 1.48
	50	6.90 $\pm$ 1.23	147.73 $\pm$ 4.01	4.61 $\pm$ 1.96	12.77 $\pm$ 0.87	64.71 $\pm$ 3.84	161.93 $\pm$ 4.26	11.23 $\pm$ 0.97
	250	6.73 $\pm$ 1.76	155.80 $\pm$ 7.01	5.65 $\pm$ 1.78	13.35 $\pm$ 0.74	73.34 $\pm$ 4.56	170.23 $\pm$ 9.16	10.23 $\pm$ 0.66
	500	6.47 $\pm$ 0.35	149.47 $\pm$ 6.66	5.99 $\pm$ 1.92	11.71 $\pm$ 0.88	71.81 $\pm$ 2.67	174.03 $\pm$ 5.14	11.96 $\pm$ 0.60
Female: the last 2 weeks	Normal	6.85 $\pm$ 1.76	152.73 $\pm$ 7.66	5.06 $\pm$ 1.29	14.51 $\pm$ 0.95	70.64 $\pm$ 1.84	164.30 $\pm$ 2.09	12.95 $\pm$ 0.83
	50	6.29 $\pm$ 0.97	154.63 $\pm$ 6.63	6.14 $\pm$ 1.21	12.69 $\pm$ 0.88	69.62 $\pm$ 1.28	177.5 $\pm$ 3.83	10.61 $\pm$ 1.01
	250	6.72 $\pm$ 1.53	152.91 $\pm$ 4.48	6.62 $\pm$ 1.43	12.70 $\pm$ 0.67	61.31 $\pm$ 4.06	169.60 $\pm$ 2.19	11.83 $\pm$ 1.87
	500	5.73 $\pm$ 1.89	153.33 $\pm$ 5.45	5.97 $\pm$ 1.18	13.6 $\pm$ 1.03	77.84 $\pm$ 1.27	168.36 $\pm$ 2.77	12.58 $\pm$ 1.19

Table 4

Blood biochemical investigation on dogs after treatment with total steroid saponins for 3 months in chronic study.

Time Interval	Parameters	Group (n=4)									
		Normal Control		50 mg/Kg		250 mg/Kg		500 mg/Kg			
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
3 rd month	ALT (U/L)	55.93 ± 4.86	58.32 ± 3.49	57.21 ± 3.83	60.36 ± 3.87	53.55 ± 3.02	56.24 ± 3.88	59.36 ± 9.03	58.01 ± 5.56		
	AST (U/L)	80.97 ± 8.92	86.22 ± 5.74	80.23 ± 7.76	78.39 ± 3.56	75.41 ± 8.42	76.42 ± 2.44	84.50 ± 6.86	82.69 ± 2.92		
	ALP (U/L)	103.43 ± 7.88	114.28 ± 6.58	117.70 ± 7.96	112.11 ± 6.64	116.01 ± 0.26	116.84 ± 4.49	116.30 ± 6.16	115.78 ± 6.79		
	TCHO (mmol/L)	3.51 ± 0.39	3.48 ± 0.32	3.35 ± 0.07	3.36 ± 0.33	3.48 ± 0.25	3.36 ± 0.42	3.61 ± 0.43	3.95 ± 0.23		
	TBIL (μmol/L)	2.52 ± 0.79	3.52 ± 0.64	2.42 ± 0.16	3.25 ± 0.56	1.76 ± 0.34	2.96 ± 0.59	2.02 ± 0.43	2.99 ± 0.69		
	TP (g/L)	81.28 ± 3.08	89.63 ± 3.01	82.42 ± 2.54	87.04 ± 2.58	80.23 ± 4.52	79.21 ± 3.10	82.65 ± 4.62	83.66 ± 4.53		
	ALB (g/L)	55.12 ± 1.76	58.98 ± 2.26	55.53 ± 1.90	57.49 ± 1.42	53.73 ± 1.33	49.54 ± 3.58	54.27 ± 1.32	52.28 ± 4.45		
	CRE (μmol/L)	76.42 ± 3.57	72.65 ± 3.62	74.53 ± 7.06	73.79 ± 4.19	79.82 ± 9.31	74.95 ± 4.15	77.75 ± 6.26	78.52 ± 4.63		
	BUN (mmol/L)	3.70 ± 0.67	3.43 ± 0.39	3.55 ± 0.47	3.18 ± 0.26	3.35 ± 0.22	3.20 ± 0.43	3.75 ± 0.65	3.32 ± 0.16		
	GLU (mmol/L)	4.25 ± 0.96	4.56 ± 0.66	4.72 ± 0.39	3.84 ± 1.88	4.68 ± 0.19	5.36 ± 1.97	4.27 ± 0.18	4.35 ± 2.73		
the last 2 weeks	ALT (U/L)	54.95 ± 1.91	56.94 ± 3.55	54.50 ± 4.38	52.50 ± 5.06	51.40 ± 2.55	54.18 ± 4.60	50.40 ± 8.20	52.39 ± 3.97		
	AST (U/L)	83.45 ± 12.52	82.24 ± 3.94	86.65 ± 3.61	79.38 ± 6.01	82.20 ± 4.81	84.18 ± 4.92	80.30 ± 1.17	81.99 ± 4.27		
	ALP (U/L)	110.80 ± 7.80	114.47 ± 3.29	107.75 ± 3.16	110.99 ± 7.92	107.35 ± 7.14	113.23 ± 7.76	102.25 ± 2.90	109.11 ± 5.55		
	TCHO (mmol/L)	3.48 ± 0.37	3.59 ± 0.28	3.45 ± 0.55	3.47 ± 0.15	3.14 ± 0.11	3.61 ± 0.19	3.26 ± 0.30	3.69 ± 0.48		
	TBIL (μmol/L)	2.33 ± 0.58	2.63 ± 0.11	2.24 ± 0.85	2.34 ± 0.88	2.27 ± 0.52	2.12 ± 0.47	2.30 ± 0.68	2.47 ± 0.59		
	TP (g/L)	81.75 ± 1.81	87.05 ± 2.75	81.80 ± 2.26	86.42 ± 3.14	87.80 ± 9.19	78.49 ± 4.39	82.75 ± 13.65	85.74 ± 2.99		
	ALB (g/L)	51.75 ± 3.46	57.24 ± 4.98	50.50 ± 8.34	58.59 ± 3.08	52.40 ± 4.18	50.30 ± 3.15	50.60 ± 9.33	56.21 ± 3.57		
	CRE (μmol/L)	76.80 ± 0.85	74.08 ± 4.05	78.75 ± 3.85	78.66 ± 4.25	82.55 ± 10.96	76.72 ± 4.57	80.30 ± 15.41	73.57 ± 4.38		
	BUN (mmol/L)	3.65 ± 0.35	3.86 ± 0.21	3.70 ± 0.71	4.01 ± 0.19	3.55 ± 0.5	3.14 ± 0.22	3.85 ± 0.78	3.14 ± 0.36		
	GLU (mmol/L)	4.55 ± 0.13	5.56 ± 1.81	4.68 ± 0.16	4.81 ± 1.45	4.54 ± 0.40	5.85 ± 0.61	4.63 ± 0.16	4.55 ± 1.85		

Table 5

The urine routine analysis of dogs fed with total steroid saponins for 3-month period in chronic toxicity evaluation.

Time Interval	Parameters	Group									
		Normal Control		50 mg/Kg		250 mg/Kg		500 mg/Kg		Male	Female
		Male	Female	Male	Female	Male	Female	Male	Female		
3 rd month	GLU (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	BIL (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	KET (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	PRO (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	URO (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	UNIT (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	UBLO (mmol/mL)	0	0	0	0	0	0	0	0	0	0
the last 2 weeks	pH	6.40 ± 1.08	9.83 ± 0.68	6.27 ± 0.96	6.49 ± 0.55	6.32 ± 0.96	6.72 ± 0.79	6.22 ± 0.70	6.86 ± 0.57		
	GLU (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	BIL (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	KET (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	PRO (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	URO (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	UNIT (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	UBLO (mmol/mL)	0	0	0	0	0	0	0	0	0	0
	pH	6.65 ± 1.48	9.86 ± 0.61	6.80 ± 0.50	6.38 ± 0.82	6.35 ± 0.21	6.32 ± 0.48	6.55 ± 0.21	6.81 ± 0.45		
	GLU (mmol/mL)	0	0	0	0	0	0	0	0	0	0