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Original Research Article

Toxicity Evaluation of Ethanol Extract of *Lansium domesticum* cv kokossan Seeds in Female Wistar Rats

Tri Mayanti[1], Darwati Darwati[1], Jamaludin Al Anshori[1], Unang Supratman[1,5], Madihah Madihah[2], Ronny Lesmana[3,5], Deden I. Dinata[4], Shabarni Gaffar[1]*

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences Universitas Padjadjaran, Sumedang, West Java,, Indonesia*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang, West Java, Indonesia*
[3]*Departement of Biomedical Science, Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia*
[4]*Bandung School of Pharmacy, Bandung, Indonesia*
[5]*Central Laboratory, Universitas Padjadjaran, Sumedang, West Java, Indonesia*

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ABSTRACT

The seed extract of *Lansium domesticum* cv kokossan contains a new tetranotriterpenoid compound, kokosanolide A, that has the potential to be used as an anticancer agent. This study aims to evaluate the acute toxicity level of the ethanol extract of *L. domesticum* cv kokossan seed in female Wistar rats. The experiment was done based on OECD 425:2006 guidelines. The rats were divided into six groups, the amount of ethanol extract of *L. domesticum* cv kokossan seeds that were given to group I-IV were 5, 50, 500 and 5000 mg/kg BW, respectively. Group V was DMSO as vehicle control and group VI was doxorubicin as a positive control. The results showed a strong negative correlation between dose of the extract and the weight gain of tested animals (r = -0.93). The administration of the extract increased the relative weight of organs only at a high dose. Histopathological examination showed a sublethal manifestation of cell damage in the form of vacuolization of hydropic and fatty acid degeneration, particularly in hepatocytes of liver and renal tubules cells. The toxic signs, organ damage and liver enzyme biomarkers did not show significant difference compared to the control. The *pseudo* LD_{50} value estimated by Probit analysis was above 5000 mg/kg BW for ethanol extract, which categorized as relatively harmless. The findings showed non-toxic effects of the ethanol extracts of *L. domesticum* cv kokosan seeds on the female Wistar rat after acute administration.

Keywords: Acute toxicity, Ethanol extract, *Lansium domesticum* seed, Rats.

Introduction

Extensive exploitation of herbal medicines has been carried out to deal with various diseases. The use of herbal medicines has been traditionally perpetuated by people who believe in them as drugs that are free of side effects.[1] However, consumers have not obtained adequate information about medicinal herbs and most of the information is not supported by scientific data.[1,2] Herbal medicine contains numerous active molecules that have the potential for both benefit and harm. Therefore, only after understanding more of their mechanisms and clinical effects, herbal medicine can be helpful to users. Studies on the efficacy and safety of medicinal plants are the main focus of researchers, due to the increasing use of natural herbal medicines.[3,4]

The biological and pharmacological properties of many plants have been examined by researchers around the world in the past few decades. These include toxicological studies for data profiling and safety of herbal medicines.[5] It is intended that modern science can take advantage of traditional medical practices and prevent the dangers that can be caused by the use of herbal medicines. Many pharmaceutical drugs come from natural sources. So, it is very important to report their toxicological effects such that the dangers of using herbal medicines can be identified and the risks of the drugs can be controlled. Scientific justification and evidence for the safety and efficacy of herbal medicines can be provided through toxicological studies.[6,7]

Acute oral systemic toxicity test is a way to determine the initial assessment and evaluation of toxic characteristics and manifestations of herbal medicines. Possible health hazards arising from short-term exposure to herbal medicines will be identified through this test. It uses a single dose of certain herbal medicines for administration to each animal on one occasion to evaluate general toxic signs and median lethal oral dose (LD_{50}). An acute toxicity test is also the basis for determining dosage levels and designing further toxicity tests.[8]

The plant of *Lansium domesticum* Corr. (Meliaceae family) is widely distributed in Southeast Asian countries. This species has three cultivars in Indonesia, namely duku, kokosan, and pisitan.[9] Phytochemical investigations of *L. domesticum* Corr. revealed the presence of several types of triterpenoids,[10-16] which possess interesting biological activities such as anticancer,[17] antibacterial,[18] insecticidal,[19] antimalarial,[20] and antifeedant activities,[21,22] and had been used in cosmetics.[23] During the search for bioactive natural products from Indonesian Meliaceae plants, we investigated two tetranortriterpenoids, kokosanolide A and C and three onoceranoid-type triterpenoids including, kokosanolide B, 8,14-secogammacera-7,14-diene-3,21-dione and 8,14-secogammacera 7,14(27)-diene-3,21-dione from the seeds and the bark of the species kokossan.[21] Seeds of *L. domesticum* have some limonoids, dukunolide A-F[24,25] and domesticulide A-E.[20] Furthermore, the leaves of *L. domesticum* cv kokossan contain Cyclolanost-24-en-3-one, 21,23-epoxy-21,22-dihydroxy (21R, 22S, 23S).[26] This study was aimed to evaluate the acute toxicity levels of the ethanol extract of *Lansium domesticum*

*Corresponding author. E mail: shabarni.gaffar@unpad.ac.id
Tel: +62-22-7794391

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Corr cv kokossan seeds which will provide information regarding the extract safety for further evaluation of biological activities and mechanisms of action of the compounds isolated from *Lansium domesticum* Corr cv kokossan.

Materials and Methods

Collection of plant materials

The fruits of *L. domesticum* cv kokossan were collected from Gede Bage (GPS coordinate: 6.9506°S, 107.6985°E), Bandung, West Java Province, Indonesia in March 2018. The plant was identified at the Laboratory of Plant Taxonomy, Department of Biology, Padjadjaran University, Indonesia. The specimen voucher (No. 10188) was deposited at the herbarium of the Padjadjaran University.

Extract preparation

The seeds of *Lansium domesticum* cv kokossan were air-dried under shade for seven days at room temperature (~26^{o}C). The dried seeds were ground by using a Blender (Philips, Japan) to fine powder. The dried seeds powder of *Lansium domesticum* cv kokossan (5 kg) were extracted successfully with 3 L of ethanol 96% at room temperature. Evaporation resulted in the crude extracts of ethanol (265 g). The crude extracts were weighed and dissolved in dimethyl sulfoxide DMSO.

Source and housing conditions of experimental animals

Wistar rats used in this experiment were healthy young adult females (10-12 weeks old), nulliparous, non-pregnant, and weighed between 150 and 250 g. The rats were obtained from Biofarma, Lembang, Indonesia. Female rats were chosen because they are slightly more sensitive than the male. Rats were randomly assigned as control and treated groups. They were housed under a standard room temperature environment with a constant relative humidity under 12-h light/dark cycles. The animals were fed with a standard laboratory pellet diet with tap water *ad libitum*. The animals were treated after a one-week adaptation in the animal room. The study was performed after getting approval from Animals Ethics Research Guidelines of Biosystem Laboratory, Biology Department, Universitas Padjadjaran. Ref. No. Bio/RIP/050119, October 2017.

Acute oral toxicity assay

The up and down procedure of acute oral toxicity assay was adapted from Economic Co-operation and Development (OECD) 425 Guidelines.[27] The animals were fasted from the food and water for 12 h before extract administration. The extract solution was administered orally by gavage using a ball-tipped stainless-steel feeding needle. The administered volume of the extract solution did not exceed 2 mL per kg body weight.

The test consists of a single ordered dose progression in which animals were treated, one at a time, at a minimum of 48-hour intervals. In the main test, the doses were 5000, 500, 50, 5 mg/kg BW, respectively. The negative control group was treated with DMSO (99.9%), and the positive control group was treated with doxorubicin (0.4 mg/kg BW). Each group of every dose consisted of six animals. After the extract administration, the animals were fasted for further 3-4 hours.

The animals were observed individually at least once during the first 30 minutes after extract administration. Subsequent observations were conducted periodically within the first 24 hours, with more attention in the first 4 hours. After 24 hours, observations were conducted daily for 14 days. All observations included visual inspections of mortality, behavioural pattern, physical appearance change, injury, pain, and the sign of illness. The animals that survive were weighed daily during the period of observations.

On the 15th day, after overnight fasting, the survived animals were sacrificed by cervical dislocation. Gross necropsies were performed to randomly selected animals, including those that die during the test. The positions, shapes, sizes, and colours of internal organs were evaluated. Heart, liver, spleen, ovaries, and kidneys were removed from all animals for the detection of gross lesions. All tissues were fixed in Bouin's solution and tissue processing was carried out in paraffin wax. Thin-sectioned tissues (5 µm) were prepared and stained with hematoxylin and eosin for histopathological examination on a microscope Olympus CX31 (Shinjuku-ku, Tokyo, Japan) with total magnificent 400× (ocular lens 10× and objective lens 40×) equipped with digital microscope Olympus E330 (Shinjuku-ku, Tokyo, Japan).

Statistical analysis

The LD_{50} was determined using probit analysis. Weight gain, the relative weight of the organ, and percentage of necropsies cells data were expressed as mean ± standard deviation (SD). Paired sample t-test at 95% confidence level was carried out using the software Statistical Package for Social Sciences (SPSS). The histopathological images were analyses with ImageJ software for Windows. The liver enzymes biomarkers, SGPT (Serum Glutamate Pyruvate Transaminase) and SGOT (Serum Glutamate Oxaloacetate Transaminase) were determined following the procedure of quantitative determination of GOT/AST (Labkit, Chemelex s.a, Barcelona) using a fully automated clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany).

Results and Discussion

According to the acute oral toxicity test, the ethanol seeds extract of *L. domesticum* cv kokosan did not caused the death of rats up to a dose of 5000 mg/kg body weight (Table 1). The probit analysis suggests that the extract is relatively safe since its *pseudo* LD_{50} is above 5000 mg/kg. The toxic signs observed after high dose administration may indicate the side effects of an extract. In our study, no test animals died between 0-14 days, therefore the *pseudo* LD_{50} value was above the maximum dose of 5000 mg/kg BW. The key toxic signs observed were a decrease of motoric activity, convulsion, salivation, and polyuria in the positive control. These toxic signs may reflect that the extract affects the nervous system, particularly the somato-muscular system, spinal integrity in the central nervous system, and the autonomic nervous system.[27,28]

Table 1: Mortality and toxic signs of female Wistar rats in the acute toxicity test of ethanol extract from *L.domesticum* cv *kokossan* seeds

Extract dose (mg/kg BW)	Number of tested animal	Number of death	Toxic signs[#]
5000	6	0	-
500	6	0	-
50	6	0	-
5	6	0	-
Doxorubicin	6	0	+ (1/4)
DMSO	6	0	-

Note: Toxic signs observed were decreasing motoric activity, convulsion, and salivation with ratio number of test animal showed toxic signs per total tested animal in a group.

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The changes of body weight were also observed, which might be an indicator of adverse side effects of a drug or extract. Both treated and control groups exhibited weight gains at the end of the acute oral toxicity test. The weight gain of treated groups, however, was lower compared to the control group (Table 2). It has a strong negative correlation with the extract dose (r = -0.93), where the higher dose decreases weight gain. Paired T-test analysis showed that P-value was greater than 0.05 (supplementary information) which therefore indicate that the dose did not have a significant effect on weight loss.

Decreasing weight gain of the treated groups may not only be caused by physiological variations such as food intake and metabolism,[29,30] but may also be due to the active compounds in the extract of *L. domesticum* cv kokosan seeds. As reported previously, the extract contains a limonoid, kokosanolid A,[21] which has shown a strong cytotoxic activity against MCF-7 breast cancer cells. Our result is consistent with the study conducted by Gatsing *et al*. (2010),[31] which showed that the acute exposure of the ethanol extract of *Alchornea cordifolia* leaves which contains triterpenes, flavonoids, saponins, anthocyanins, polyphenols, steroids, and tannins, at doses of 2000-16000 mg/kg body weight showed a lower weight gain of the treated groups compared to the control groups.[31]

Another essential part of this study is organ weight analysis. Organ weight is the most sensitive indicators of an effect of the test substance. And relative organ weight is crucial for the physiological and pathological status of animals, to diagnose whether the treatment injure the organs.[32,33] The weight of organs in the extract treated groups were lower compared to either negative or positive control (Table 3). Alterations of the weight of the vital organs may suggest treatment-related changes including hypertrophy.[34] The extract administration did not show significant weight differences of the organs including liver (p-value = 0.108), spleen (0.074), right kidneys (0.552), heart (0.192), and right ovary (0.652) (Supplementary information), which indicated that there was no significant effect of the extract treatment on the organ weight gain. Relative organ weight is crucial for the physiological and pathological status of animals, to diagnose whether the treatment injure the organs.[30-32] The liver plays a major role in metabolism, including regulation, decomposition of red blood cells, protein synthesis, hormone production, and detoxification.[33, 34]

Changes in organ weights should always be interpreted in conjunction with necropsy and histopathological findings because of the inherent variability.[37,38] Necropsy and histopathological examinations can reveal the major target organs for toxicity and may help to direct subsequent testing.[37] Necropsy and histopathological examinations can confirm any damage in organs or tissues. The necropsy examinations of the tested animal organs did not show any changes in colour and texture of all organs compared to the controls (positive/negative control group).

Nevertheless, histopathological examination of the liver and kidney showed a slight disruption in tissue architecture of the treated animals (Table 4), whereas the heart, spleen, and ovaries showed normal architecture (Figure 1). On microscopic examinations, vacuolization in the form of hydropic and fatty acid degeneration were observed and categorized as sub lethal manifestation of cell damage, although it was a reversible abnormality.[38,41,42] These types of cell damages were common in cell with high rate of metabolism, such as in hepatocytes and proximal tubular cells and may be categorized as a slightly toxic effect.[39] The spleen shows normal cellularity and size of the red and white pulp. The heart histology showed normal cardiac myocytes in elongated shapes. The ovary histology showed normal stages and size of egg follicle, with no atresia follicle. The necropsy and histopathological examination is paramount in linking the general and target organ specific in the toxic effects of phytomedicine.[27] In this study, however, the extract treatment may not cause adverse effect on the organ histology.

The distortion of the cyto-architecture of the liver and dilatation in the central vein as observed in the higher dose administration was not associated with functional changes of the liver that may be detrimental to the health of the test animals. The results of the hepatic enzymes markers (SGOT and SGPT) showed no significant different from the control. However, slight increase in SGPT value and lower value of SGOT were observed at all doses of the extract treatment compared with the control (Table 5).

Our previous research also shows that ethanol extract of *Lansium domesticum* cv kokossan leaves was relatively harmless to female Wistar rats with LD_{50} = 16538.49 mg/kg BW.[43] These preliminary results suggested that *Lansium domesticum* cv kokossan should be further evaluated for long term use and repeated dose effects to ensure safety of this herb.

Table 2: Body weight of female Wistar rats in the acute toxicity test of the ethanol extracts from *L. domesticum* cv *kokossan* seeds

Extract dose (mg/kg BW)	Body weight (g)		
	Day 1	Day 14	Weight Gain (g)
5000	219.00 ± 13.99	214.33 ± 35.02	(-4.7) ± 27.13
500	221.33 ± 13.81	211.17 ± 8.35	(-10.2) ± 17.54
50	216.17± 14.57	219.67 ± 20.76	3.5 ± 9.16
5	213.67 ± 11.66	219.33 ± 15.10	5.7± 5.85
Doxorubicin	196.17 ± 10.38	188.67 ± 27.41	(-7.5 ± 20.02)
DMSO	221.17 ± 8.23	208.50 ± 16.44	(-12.7) ± 13.43

Data are expressed as mean ± SD (n=6). Data analyses by paired-sample t-test with 95% confidence level.
*p < 0.05 - significantly different with respect to the control group.

Table 3: Relative weight of organ of female Wistar rats in the acute toxicity test of the ethanol extracts from *L. domesticum* cv *kokossan* seeds

Extract dose (mg/kg BW)	Liver (%)	Heart (%)	Right kidney (%)	Spleen (%)	Right ovary (%)
5000	3.92 ± 0.399	0.36 ± 0.039	0.38 ± 0.154	0.35 ± 0.049	0.28 ± 0.106
500	3.49 ± 0.522	0.41 ± 0.059	0.39 ± 0.049	0.34 ± 0.05	0.27 ± 0.158
50	3.62 ± 0.371	0.37 ± 0.016	0.38 ± 0.064	0.29 ± 0.066	0.28 ± 0.078
5	3.60 ± 0.536	0.39 ± 0.067	0.39 ± 0.029	0.30 ± 0.029	0.25 ± 0.048
Doxorubicin	4.00 ± 0.063	0.36 ± 0.055	0.39 ± 0.043	0.26 ± 0.044	0.30 ± 0.065
DMSO	4.20 ± 0.445	0.43 ± 0.069	0.45 ± 0.04	0.34 ± 0.087	0.35 ± 0.134

Data are expressed as mean ± SD (n = 6). Data analyses by paired-sample t-test with 95% confidence level. *p < 0.05 - significantly different with respect to the control group.

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Table 4: Histopathological effects of the extract

Treatment (mg/kg body weight)	Histological effect on liver tissue	Histological effect on kidney tissue	Histological effect on heart tissue	Histological effect on spleen tissue	Histological effect on ovaries tissue
5000	Dilatation of central vein, normal sinusoids, and an increased of cell degeneration in hepatocyte.	Renal corpuscle showed normal, and an increase of degenerative changes in the tubules.	Myocardium appeared normal. No evidence of degenerative tissue changes.	White and red pulp appeared normal in size. Lymphocyte and macrophage population normal.	Follicles appeared and normal. No evidence of atretic follicles.
500	Dilatation of central vein, normal sinusoids, and a few of cell degeneration in hepatocyte.	Renal corpuscle showed normal, and an increase of degenerative changes in the tubules.	Myocardium appeared normal. No evidence of degenerative tissue changes.	White and red pulp appeared normal in size. Lymphocyte and macrophage population normal.	Follicles appeared and normal. No evidence of atretic follicles.
50	Central vein and sinusoids appeared normal; a few of cell degeneration in hepatocyte.	Renal corpuscle showed normal, but there are degenerative changes in the tubule.	Myocardium appeared normal. No evidence of degenerative tissue changes.	White and red pulp appeared normal in size. Lymphocyte and macrophage population normal.	Follicles appeared and normal. No evidence of atretic follicles.
5	Central vein and sinusoids appeared normal; cell degeneration in hepatocyte.	Renal corpuscle and tubules appeared normal.	Myocardium appeared normal. No evidence of degenerative tissue changes.	White and red pulp appeared normal in size. Lymphocyte and macrophage population normal.	Follicles appeared and normal. No evidence of atretic follicles.
Doxorubicin	Central vein, sinusoids, and hepatocytes appeared normal.	Renal corpuscle and tubules appeared normal.	Myocardium appeared normal. No evidence of degenerative tissue changes.	White and red pulp appeared normal in size. Lymphocyte and macrophage population normal.	Follicles appeared and normal. No evidence of atretic follicles.
DMSO	Central vein, sinusoids, and hepatocytes appeared normal.	Renal corpuscle and tubules appeared normal.	Myocardium appeared normal. No evidence of degenerative tissue changes.	White and red pulp appeared normal in size. Lymphocyte and macrophage population normal.	Follicles appeared and normal. No evidence of atretic follicles.

Table 5: Effect of Ethanol Extract of *Lansium domesticum* cv *kokossan* Seeds on SGPT and SGOT as hepatic function markers of rats after acute administration (n = 6)

Extract doses (mg/kg BW)	SGPT	SGOT
Control	1.12 ± 0.09	17.21 ± 1.70
Doxorubicin	7.98 ± 0.78	17.66 ± 1.84
5000	7.49 ± 1.66	14.8 ± 0.62
500	7.76 ± 0.5	15.98 ± 1.51
50	7.28 ± 0.78	15.08 ± 1.68
5	5.99 ± 0.61	16.55 ± 2.29

Data are expressed as mean ± SD (n=6). The t-test at α= 0.05 showed no statistically significant differences between doses within each marker.

Conclusion

The findings showed a non-toxic effects of the acute administration of the ethanol extracts of *L. domesticum* cv kokosan seeds at doses between 5 - 5000 mg/kg body weight, particularly on the body weight gain, the vital organ weight, histological appearance, and hepatic enzymes. This study suggests that *L. domesticum* cv kokosan seeds extract and their preparations is relatively safe.

Conflict of interest

The authors declare no conflicting interest

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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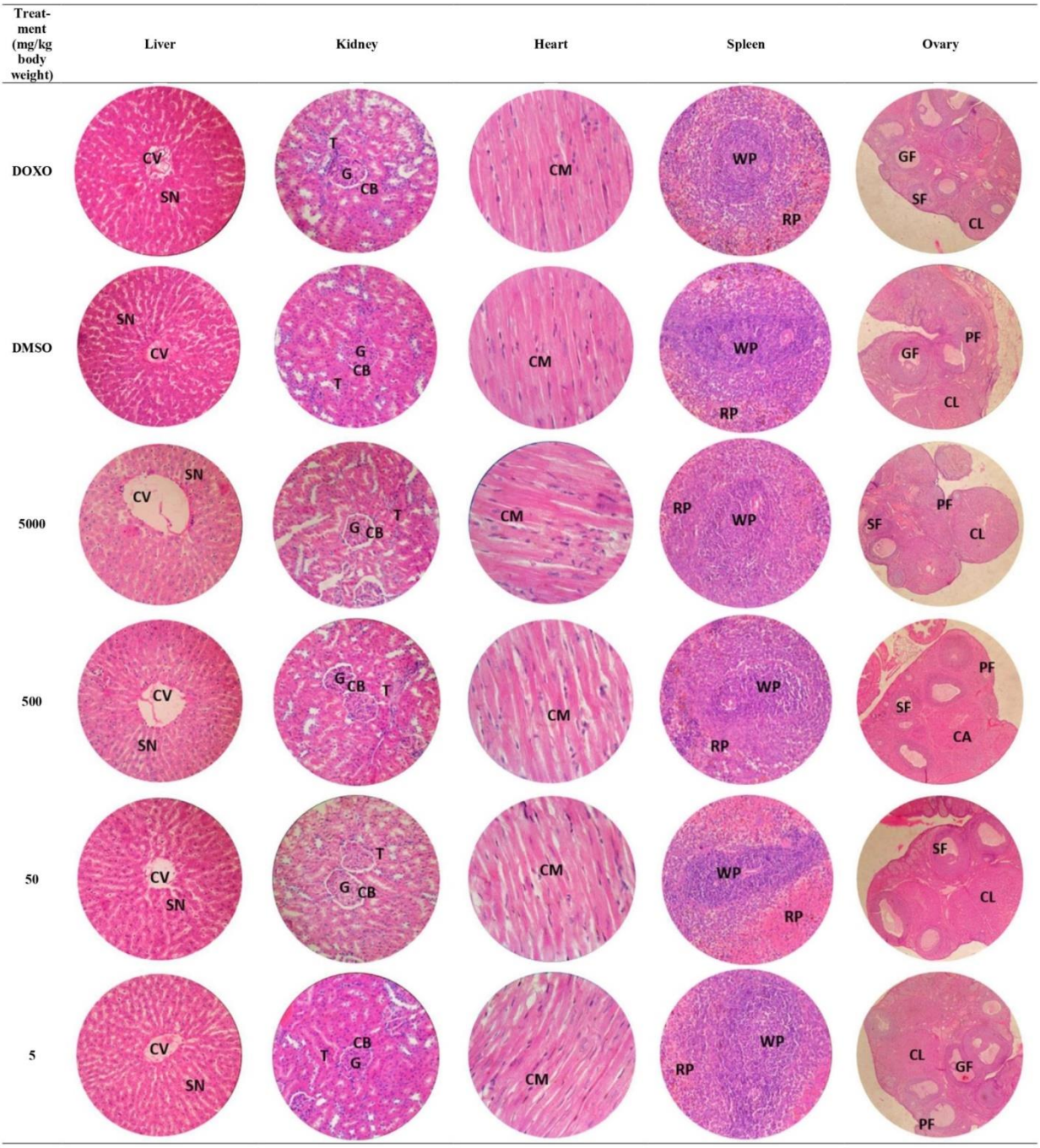

Figure 1: Photomicrograph of the tissue cross section. Note: **CV.** Central Vein; **SN.** Sinusoid; **G.** Glomerulus; **CB.** Capsule of Bowman: **T.** Renal tubule; **CM.** Cardiac myocyte; **RP.** Red pulp; **WP.** White pulp; **PF.** Primary follicle; **SF.** Secondary follicle; **GF.** Graffian follicle; **CL.** Corpus luteum; **CA.** Corpus albicans. Arrowhead. Vacuolisation in form of hydropic or fatty degeneration; Arrow. Dilatation in central vein

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