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Original Research Article

The Repeated Dose 28-Day Oral Toxicity Study of Combined Extract of *Cajanus cajan* Leaf and *Zingiber officinale* Rhizome in Male and Female Sprague-Dawley Rats

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ABSTRACT

The combination of *Cajanus cajan* leaf and *Zingiber oficinale* rhizome extract resulted in a greater hypoglycemic effect than the single of both extracts. However, no research reported the toxic effect of both extracts. So, this study assessed the toxicity of a combination of *C. cajan* leaves and *Z. officinale* extract in male and female Sprague-Dawley rats. Thirty male and thirty female rats were used in this study. The rats were randomly assigned to six groups of ten rats per group (five males and five females): the control treatment group and three-dose extract treated groups for 28 days-treatment periods. The satellite control group and satellite group were extended for 14 days-recovery periods. The combination doses of *C. cajan* leaves extracts, and *Z. officinale* extract were 200+100, 400+200 and 800+400 mg/kg bw. The number of dead rats, body weight, biochemistry, hematology, and histopathological observation of some organs were observed. The results demonstrated that orally administering the combination of *C. cajan* and *Z. officinale* extracts for 28 day did not show any toxic impacts on male and female rats. Moreover, no cumulative effects of the combined extracts were noted during the 14-day recovery period. These findings showed that there were insignificant alterations in body weight, hematology, biochemistry, and histopathological observations. The findings suggest the safety of the combined extract of *C. cajan* leaf and *Z. officinale* rhizome.

Keywords: *Cajanus cajan*, *Zingiber officinale*, repeated dose 28-day oral toxicity study, biochemistry, histopathology

Introduction

Cajanus cajan, also known as "pigeon pea" in English or "Undis" in Indonesia, is a shrub that grows in the lowlands. *C. cajan* is widely planted in Indonesia in West Nusa Tenggara.[1] It has many medicinal properties, such as anti-inflammatory, anti-microbial, antibacterial, antidiabetic, hypocholesterolemic effects, anti-cancer, neuroactive properties, and antioxidants.[2] *Zingiber officinale*, known as "ginger" in English or "Jahe" in Indonesia, is a rhizome plant that belongs to the Zingiberaceae family and is a perennial herb with rhizomes.[3] It is commonly used as a cooking spice and medicinal plant. The pharmacological effects of ginger include analgesic, anti-inflammatory, antifungal, antibacterial, anti-ulcer, and immunomodulatory.[4]

The combination of *C. cajan* leaf and *Z. oficinale* rhizome extract resulted in a greater hypoglycemic effect than the single of both extracts. The combination formulation of *Cajanus cajan* and *Zingiber officinale* has also been reported to be effective as an antioxidant, hypoglycemic activity, and antidiabetic effect.[5,6]

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The antioxidant properties of *C. cajan* and *Z. officinale* exhibited were shown in the following parameters: DPPH-free radical scavenging assay; increased Cu,Zn-SOD antioxidant content in the testis, pancreas, liver, and kidney; increasing total superoxide dismutase (SOD) and lowering malondialdehyde (MDA) in liver and kidney.[6,7] Additionally, the antidiabetic efficacy of both extracts was showed by the following effect: inhibiting α-glucosidase activity, increasing body weight, lowering blood glucose levels, inhibiting pancreatic β-cells damage, increasing serum insulin levels, and increasing the neutral carbohydrates content in the liver and muscle.[5,7] Administration of these extracts increased enhanced spermatogenic and interstitial cell count and inhibited kidney function damage.[6,7] *C. cajan* and *Z. officinale* extracts also have an antihyperlipidemic effect, inhibiting HMG-CoA reductase activity.[8]

Although some efficacy studies of both extracts have been reported, there are limited investigation into the safety aspects of this medicinal plant. Previous research reported that acute toxicity of *C. cajan* using ethanol 96% as the solvents showed a 50% lethal dose (LD_{50}) >5000 mg/kg bb and was classified as practically nontoxic.[9] In repeated dose 28-day oral toxicity study, the administration of *C. cajan* extract in rats over 28-day treatment and 14-day recovery periods did not change hematological and biochemical parameters and histopathology organs at the dose of 3 and 6 g/kg bw.[10] In addition, the toxicity test of *Z. officinale* demonstrated that the 50% lethal concentration (LC_{50}) was 71.01 ppm in methanol, 63.81 ppm in n-hexane fraction, and 3821.89 ppm in ethyl-acetate fraction.[11] However, no research reported the safety aspect of the combination of *C. cajan* and *Z. officinale*. So, the current study assessed the repeated dose 28-day oral toxicity study of the combination of *C. cajan* and *Z. officinale* in rats. The toxicity study is (1) to provide data on potential toxic effects after following repeated exposure to the test combination over 28 day-treatment periods; (2) to provide non-effect dose information on toxicity (No Observed Adverse

Effect Level); and (3) to determine the existence of cumulative and reversible effects of the combination for 14-day recovery periods.

Materials and Methods

Materials

Cajanus cajan (L.) Huth leaves were obtained from Lombok, West Nusa Tenggara, Indonesia on July, 2022. *Zingiber officinale* var. amarum rhizome was obtained from Solo, Central Java, Indonesia on July, 2022. *C. cajan* and *Z. officinale* were identified and stored in Tropical Biopharmaca Research Center (Trop BRC), IPB University, Bogor Indonesia with voucher specimen numbers: BMK0515072022 and BMK0388012018, respectively.

Plant extraction

The dried *C. cajan* leaves and *Z. officinale* rhizome were ground and mashed, then sifted with a size of 40 mesh, separately. The process was conducted at Trop BRC. The powder of *C. cajan* leaves and *Z. officinale* rhizome was extracted separately using maceration in 70% ethanol at 1:10 (powder: solvent) for 24 hours. The maceration filtrate was subjected to evaporation using a vacuum evaporator at 70 °C with addition of the filler: amylum (Chargill Bio-Chemical Co., ltd) and aerosil (WACKER). The *C. cajan* and *Z. officinale* extracts contained 35% dry extract (DE) and 65% filler (amylum).

Experimental Animals

The study used male and female rats (8-12 weeks; *Sprague Dawley*) with an average maximum weight variation of 20% (200±10 g). Sixty rats were used, including 30 males and 30 females. The female animals used were healthy and mature, had never given birth and were not pregnant. Rats were purchased from the Food and Drug Supervisory Agency-Republic of Indonesia (BPOM-RI). The rats were acclimatized for seven days.

The rat husbandry room was set at a temperature of 22°±3° C, 55-65% relative humidity, 12 hours of light and 12 hours of darkness, and kept from the noise. The room always was kept clean. Rats were given standard feed and drank *ad libitum*. The cage size referred to the Guide for the Care and Use of Laboratory Animals (2011), National Research Council (US) Committee. Each rat was kept in a cage with dimensions: 47x34x20 cm. Each cage was equipped with bedding from wood sawdust after a sterilization process. The bedding of the cage was changed twice a week. The Animal Ethics Committee of the Faculty of Veterinary Medicine at IPB University granted approval for the procedure, with the assigned number 040/KEH/SKE/X/2022.

Repeated Dose 28-day Oral Toxicity Study

The repeated dose 28-day oral toxicity study used 60 rats (30 males and 30 females) which were divided into six groups (four treatment groups and two satellite groups).[13,14] Each group consisted of five males and five females. The six groups were as the following:

Treatment groups

Group 1: Control group rats were given water
Group 2: rats were given combination 200+100 mg/kg bw of *C. cajan* and *Z. officinale* extract
Group 3: rats were given combination 400+200 mg/kg bw of *C. cajan* and *Z. officinale* extract
Group 4: rats were given combination 800+400 mg/kg bw of *C. cajan* and *Z. officinale* extract
Satellite groups:
Group 5: Control satellite group rats were given water
Group 6: rats were given combination 800+400 mg/kg bw of *C. cajan* and *Z. officinale* extract

C. cajan leaves extract and *Z. officinale* extracts were dissolved together in water, and tween (80%) was added as a surfactant in the amount of 1:1000 of the water solvent. The treatment group was given the extracts orally for 28-day periods, then sacrificed on the 29th day. The satellite group was given the extract orally for 28 days, then stopped on the 29th to 42nd day (14-day recovery period). On day 43, the rats were sacrificed.

Table 1: Histopathological damage score of the organ due to *combined extract of Cajanus cajan and Zingiber officinale*

Organ	Score	Description
Brain	0	No abnormalities
	1	Mild neuronal degeneration (edema)
	2	Moderate neuronal degeneration (pyknosis)
	3	Necrosis of neuronal cells with satelliteosis
Lungs	0	No abnormalities
	1	Mild alveolar walls damage
	2	Moderate alveolar walls damage
	3	Severe damage to alveolar necrosis
Heart	0	No abnormalities
	1	Mild damage of heart muscle (loss of striation)
	2	Moderate damage of heart muscle (muscle Zenker's degeneration)
	3	Cardiac muscle necrosis
Liver	0	No abnormalities
	1	Zone 1 mild degeneration (proliferation of bile duct and anisocytosis of liver cells)
	2	Zone 2 moderate degeneration (hydropic to fat degeneration)
	3	Degeneration to diffuse necrosis
Kidney	0	No abnormalities
	1	Mild degeneration of proximal tubules and normal mesangial cells
	2	Moderate degeneration of the proximal tubule, mild degeneration of the mesangium
	3	Degeneration up to tubular necrosis, edema and necrosis of mesangium

Spleen	0	No abnormalities
	1	Mild depletion of white pulp
	2	Moderate depletion of white pulp
	3	Severe depletion of white pulp
Pancreas	0	No abnormalities
	1	Mild degeneration of the islets of Langerhans cells
	2	Degeneration and edema of the islets of Langerhans cells
	3	Widespread damage to the islands of Langerhans and Acinar
Ovary	0	No abnormalities
	1	Mild damage to primary follicle
	2	Moderate damage (theca cell necrosis)
	3	Egg necrosis of follicle
Testis	0	No abnormalities
	1	Mild damage of tertiary spermatids
	2	Moderate damage of secondary and tertiary spermatids until there are no spermatozoa
	3	Necrosis of primary spermatocytes or basal layer

Blood Sampling and Sacrificing
Rats were fasted for 10 hours before blood collection and sacrifice. On day 0, blood in the treatment group and satellite was collected via the caudal vein as a baseline. Rats were euthanized on the 29th day (treatment groups) and the 43rd day (satellite groups). Anaesthetic used was ketamine (70 mg/kg bw) and xylazine (10 mg/kg bw) intraperitoneally (i.p). After being unconscious, blood was collected intracardially. Rats were dissected and exsanguinated. Organs were collected and fixed in Bouin's fixative solution for 24 hours. The organs were then transferred to 70% alcohol (stopping point).[15]

Mortality Observation and Body Weight
Animal mortalities were observed and noted for treatment and recovery period. The measurement of body weight was conducted on a weekly basis.

Hematology parameters
The hematological examination was conducted using a Hematology Analyzer. The parameters measured were hemoglobin (Hb), erythrocyte (red blood cells/RBC), leukocyte (white blood cells/WBC), hematocrit (HCT), platelet (thrombocyte), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC).

Serum Biochemistry
Blood biochemical analysis used the Selectra Junior Analyzer instrument (Vital Scientific, The Netherlands). The parameters measured were alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, cholesterol total and triglycerides.[9]

Histopathological Examination
The organs evaluated were the liver, kidney, brain, lungs, heart, spleen, pancreas, ovary and testis. Organs were processed using a histopathology routine, including trimming, dehydration, clearing, embedding, and staining (hematoxylin-eosin).[9] The HE-stained tissues were captured under a light microscope (Olympus CX31). The histopathological parameters were in accordance with the details outlined in Table 1.

Statistical analysis
The data were analyzed using an independent t-test. The Mann-Whitney test was used if the data were not eligible for an independent t-test (i.e., homogeneous and normally distributed). Data analysis was conducted using the SPSS 22.00 software.

Results and Discussion

Mortality Rats
The study showed no mortality of rats after combined extract of *C. cajan* and *Z. officinale* treatments in all groups. The administration of combined extracts in group 2, 3, and 4 for 28 day-treatment periods did not cause mortality. In addition, stopping combined extracts for 14-day recovery periods after 28-day treatment also did not cause mortality in all groups.

Effect on Body Weight
Table 2 show the body weight in treatment and satellite groups after the combined extract of *C. cajan* and *Z. officinale* treatments. At 0 days, all groups had no significant difference in body weight. However, group 3 and 4 indicated a significant weight gain on the seventh day compared to the group 1 ($p<0.05$). In satellite groups, exposure to 800+400 mg/kg bw of both extracts significantly increased body weight on the 21st day ($p<0.05$). At the end of treatment, all treatment groups had no significant change in body weight. The same results were shown in satellite groups. No significant change in body weight was found in the control and satellite groups after 14-day periods. The results indicated that both extracts in all doses did not affect body weight.
The alteration in body weight is a crucial marker for toxicity, the progression of an illness, and an individual reaction to the medication.[16] In male rats, a significant increase in body weight was found in group 3 ($p<0.05$) and group 4 ($p<0.05$) at seven days and group 6 at 21 days ($p<0.05$) (Table 2). This increase in body weight was not dose-dependent and incidental because there was no significant difference on other days. Previous research showed that male rats treated with ethanol extract of *C. cajan* at 6 g/kg bw for 28 days-treatments and 14 days recovery period significantly increased body weight. However, female rats were insignificant at 2, 4, and 6 g/kg bw.[10]

Effect on Hematology Profile
Table 3 show the blood hematology profile of male rats after the combined extract of *C. cajan* and *Z. officinale* 28-day treatment period. At 0 days, the blood hematology of rats was measured as a baseline. MCV and MCH in the group 2 increased significantly compared to the group 1 ($p<0.05$; $p<0.01$). However, MCV and MCH still were in the normal range. Significantly higher levels of RBC, Hb, and HCT were found in the group 3 and 4 compared to the group 1. Moreover, group 2 exhibited a significantly higher Hb level compared to the group 1($p<0.05$). MCHC in group 3 ($p<0.01$) and WBC ($p<0.01$) in group 2 were significantly higher than the group 1. Nevertheless, the alterations in RBC, WBC, HCT, MCHC, and WBC remained within the normal

range. (Table 3). In satellite groups, all blood haematology parameters were not detected as significantly different. These results show that alterations in hematological parameters of rats treated with all doses for 28 days were reversible after the 14-day recovery period.

Table 4 show the hematological parameters of female rats treated with the combined extract of *C. cajan* and *Z. officinale*. There were no significantly different hematological parameters at 0 days (baseline). A significantly higher level of RBC was detected in group 3 compared to group 1 ($p<0.05$). In addition, platelet levels in group 2 showed significantly lower than in group 1 ($p<0.05$). However, these RBC and platelet change levels were still in the normal range. These changes were reversible, as supported by the fact that there were no significant changes in hematological parameters in satellite groups.

All changes in hematology parameters of rats treated with both extracts still were in the normal range. Furthermore, the study revealed no hematology effect in male and female rats treated with the combined extract of *C. cajan* and *Z. officinale* for 28 days. Besides, 14-day recovery periods exhibited a reversible effect on the hematological effects of rats.

Evaluating hematological parameters is a helpful way to assess the harmful impact of foreign substances, including plant materials, on blood.[17] At the end day, RBC, Hb, and HCT in male rats after 28-day treatment periods decreased significantly in groups 3 and 4 compared to group 1 (Table 3). Nonetheless, these alterations were within normal range found in comparable species, strains, and sex. (RBC: 7 – 10x10^6 /µL; Hb: 11–18 g/dL, HCT: 36 – 48%).[18] Additionally, 14-day recovery periods in group 6 showed insignificant RBC, Hb, and HCT compared to group 5. Furthermore, these changes were reversible. Group 3 demonstrated a significant increase in MCHC to control treatment ($p<0.01$), and this condition was normal as compensation to increasing in Hb concentration in RBC.[19] In addition, group 2 decreased significantly compared to group 1 ($p<0.01$). Nevertheless, change in MCHC and RBC were not-dependent dose and within the normal range in similar species, strains, and sex (MCHC: 25.4–80.5 g/dL, WBC: 4.4–14.8x10^3/µL).[20]

In female rats, there were two significant changes in the treatment group; RBC in group 3 and platelet in group 2 as compared to group 1 (Table 4). Nonetheless, these alterations were normal in similar species, strains, and sex (RBC: 7 – 10x10^6 /µL, platelet: 100–1300x10^3 /µL).[18] Hence, these changes in hematological parameters were considered biologically insignificant and incidental.[21] So, the administration of the combined extract of *C. cajan* and *Z. officinale* for 28-day treatment did not change the hematological parameter in male and female rats. In previous results, 28-day exposure to *C. cajan* did not change hematological parameters in male and female rats.[10]

Effect on Biochemistry Profile

Table 5 show the blood biochemistry profile of male rats treated with the combined extract of *C. cajan* and *Z. officinale*. The creatinine levels in group 2 showed a significant difference compared to group 1 ($p<0.05$). However, creatinine levels in group 2 still were in the normal range. At 28 days, ALT levels in group 2 were significantly higher than in the group 1 ($p<0.05$). The levels of AST in groups 2, 3 and 4 demonstrated a significant decrease in all dose when compared to group 1 ($p<0.01$; $p<0.05$). Also, a significantly higher level of urea was detected in group 2 compared to group 1 ($p<0.05$). In the satellite group, the triglyceride level was significantly lower than in group 1 ($p<0.05$).

Table 6 show the biochemistry profile of female rats treated with the combined extract of *C. cajan* and *Z. officinale*. At 0 days, there were no significant differences in the biochemistry profile in all doses in the treatment groups. However, a significantly higher creatinine level was detected in the group 6 compared to group 5 ($p<0.05$). At 28 days, creatinine levels in group 2 and 3 were significantly higher than group 1 ($p<0.01$; $p<0.05$). The biochemistry profile in the satellite group was not significantly different.

The study revealed that all changes in the biochemistry profile of rats treated with the combined extract of *C. cajan* and *Z. officinale* still were in the normal range. Consequently, there was no biochemistry change in male and female rats treated with both extracts for 28 days. Besides, 14-day recovery periods exhibited a reversible effect on the hematological effects of rats.

Table 2: The effect of combined extract of *Cajanus cajan* and *Zingiber officinale* treatments on body weight in rats

Days	Treatment Groups				Satellite Groups	
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
Males						
0	219.78 ± 12.00	207.58 ± 15.84	200.22 ± 16.61	206.12 ± 10.68	205.84 ± 12.05	203.60 ± 2.030
7	202.26 ± 6.50	207.58 ± 15.84	215.48 ± 7.77*	228.78 ± 18.79*	243.02 ± 13.30	232.12 ± 12.57
14	251.66 ± 18.37	237.74 ± 17.27	229.96 ± 9.00	238.34 ± 17.48	241.18 ± 10.65	233.98 ± 14.39
21	283.32 ± 24.10	265.94 ± 24.29	257.3 ± 13.94	271.28 ± 24.54	241.18 ± 10.65	272.78 ± 21.92#
28	273.22 ± 21.44	268.5 ± 28.04	270.44 ± 11.72	279.22 ± 21.63	293.36 ± 19.67	274.74 ± 24.10
35					342.56 ± 16.01	328.96 ± 31.13
42					380.94 ± 13.22	364.54 ± 34.74
Females						
0	192.66 ± 18.96	191.54 ± 13.04	196.76 ± 21.86	194.96 ± 16.72	192.78 ± 14.72	193.86 ± 14.99
7	206.70 ± 16.61	206.7 ± 13.88	196.62 ± 34.48	218.26 ± 26.11	204.02 ± 12.73	204.4 ± 19.09
14	209.84 ± 24.87	206.02 ± 11.72	200.20 ± 32.50	215.84 ± 29.28	208.16 ± 19.44	200.24 ± 20.07
21	212.98 ± 15.17	220.36 ± 20.05	214.02 ± 30.35	215.00 ± 35.16	219.54 ± 11.74	215.94 ± 27.43
28	215.5 ± 18.69	223.86 ± 21.79	219.02 ± 30.14	224.1 ± 33.08	222.96 ± 11.43	216.64 ± 28.94
35					226.2 ± 11.78	221.44 ± 19.94
42					234.72 ± 5.86	231.64 ± 35.25

Group 1: water, Group 2: 200+100 mg/kg bw of *C. cajan* and *Z. officinale*, Group 3: 400+200 mg/kg bw of *C. cajan* and *Z. officinale*, Group 4: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*, Group 5: water, Group 6: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*. Data are represented as mean ± standard deviation (SD) (n=5). *$p<0.05$ Vs. Group 1 in treatment groups with Independent t-test. #$p<0.05$ Vs. Group 5 in satellite groups with Independent t-test.

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Table 3: Blood Hematology of male rats after combined extract *of Cajanus cajan and Zingiber officinale*

Days	Parameters	Treatment Groups			Satellite Groups			Normal Range	Unit
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6		
0	RBC	8.91 ± 0.99	9.50 ± 1.44	8.91 ± 1.04	10.12 ± 1.32	9.03 ± 1.36	9.68 ± 1.37	7 – 10	10^6 /µL
	Hb	16.10 ± 1.97	16.66 ± 2.72	16.24 ± 1.97	18.10 ± 2.24	16.66 ± 2.51	17.86 ± 2.66	11 – 18	g/dL
	HCT/PCV	50.20 ± 5.72	51.98 ± 7.99	50.02 ± 5.49	55.52 ± 6.80	50.84 ± 6.91	53.86 ± 7.66	36 – 48	%
	MCV	56.42 ± 1.37	54.66 ± 0.69*	56.08 ± 2.03	54.76 ± 0.83	56.36 ± 1.17	55.76 ± 0.27	48.9–57.9	fL
	MCH	18.02 ± 0.23	17.50 ± 0.20**	18.20 ± 0.43	17.90 ± 0.33	18.44 ± 0.25	18.42 ± 0.16ab	17.1– 20.4	Pg
	MCHC	32.08 ± 0.63	32.04 ± 0.36	32.48 ± 0.43	32.66 ± 0.23	32.92 ± 0.63	33.14 ± 0.28	25.4–80.5	g/dL
	Platelet	841 ± 179.52	701.80 ± 368.41	966.40 ± 186.46	836.20 ± 110.23	729.20 ± 180.65	569.40 ± 230.57	100–1300	10^3 /µL
	WBC	11.05 ± 2.35	9.25 ± 1.95	9.85 ± 4.33	11.23 ± 2.56	10.82 ± 0.81	10.46 ± 1.79	4.4– 14.8	10^3 /µL
28/42[a]	RBC	8.91 ± 0.44	8.32 ± 0.53	8.07 ± 0.32**	8.28 ± 0.33*	8.38 ± 0.13	8.28 ± 0.21	7 – 10	10^6 /µL
	Hb	15.52 ± 0.63	14.46 ± 0.74*	14.12 ± 0.25**	14.50 ± 0.61*	14.70 ± 0.52	14.54 ± 0.61	11 – 18	g/dL
	HCT	50.76 ± 4.61	45.88 ± 2.34	43.20 ± 1.05**	45.12 ± 1.78*	46.76 ± 1.77	46.78 ± 2.07	36 – 48	%
	MCV	54.72 ± 1.08	55.20 ± 1.75	53.54 ± 1.46	54.46 ± 1.66	55.78 ± 1.51	56.48 ± 1.33	48.9–57.9	fL
	MCH	17.44 ± 0.42	17.38 ± 0.50	17.50 ± 0.46	17.50 ± 0.50	17.54 ± 0.47	17.56 ± 0.32	17.1– 20.4	Pg
	MCHC	31.86 ± 0.32	31.52 ± 0.13	32.68 ± 0.29**	32.14 ± 0.55	31.44 ± 0.32	31.08 ± 0.37	25.4–80.5	g/dL
	Platelet	834.40 ± 242.31	710.80 ± 309.79	950.40 ± 21.68	965.80 ± 115.72	897.40 ± 58.38	841.60 ± 259.62	100–1300	10^3 /µL
	WBC	8.42 ± 1.62	4.32 ± 1.58**	7.60 ± 3.52	6.00 ± 2.14	7.59 ± 0.81	6.75 ± 1.27	4.4– 14.8	10^3 /µL

([a]): 28 days for treatment groups and 42 days for satellite groups. RBC: Red blood cells, Hb: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, WBC: White blood cells. Group 1: water, Group 2: 200+100 mg/kg bw of *C. cajan* and *Z. officinale*, Group 3: 400+200 mg/kg bw of *C. cajan* and *Z. officinale*, Group 4: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*, Group 5: water, Group 6: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*. Data are represented as mean ± standard deviation (SD) (n=5). *p<0.05; **p<0.01 Vs. Group 1 in treatment groups with Independent t-test

Table 4: Blood Hematology of female rats after combined extract *of Cajanus cajan and Zingiber officinale* treatments

Days	Parameters	Treatment Groups			Satellite Groups			Normal Range	Unit
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6		
0	RBC	8.45 ± 0.48	8.68 ± 0.31	8.58 ± 0.33	8.22 ± 0.56	8.38 ± 0.38	8.46 ± 0.31	7 – 10	10^6 /µL
	Hb	15.28 ± 0.59	15.42 ± 0.38	15.34 ± 0.81	14.78 ± 0.61	15.00 ± 0.45	15.18 ± 0.21	11 – 18	g/dL
	HCT	46.02 ± 1.71	47.1 ± 1.58	46.08 ± 2.53	44.74 ± 1.87	45.00 ± 1.63	46.26 ± 1.08	36–48	%
	MCV	54.54 ± 1.53	54.28 ± 1.42	53.64 ± 1.07	54.52 ± 1.65	53.68 ± 1.40	54.68 ± 1.13	53–59.5	fL
	MCH	18.10 ± 0.72	17.80 ± 0.46	17.86 ± 0.38	18.00 ± 0.54	17.90 ± 0.41	17.98 ± 0.51	18.3– 20	Pg
	MCHC	33.18 ± 0.43	32.80 ± 0.51	33.30 ± 0.64	33.04 ± 0.27	33.32 ± 0.52	32.80 ± 0.48	32.7–35.7	g/dL

	Platelet	1002.6 ± 118.71	1012.4 ± 327.68	781.00 ± 331.33	943.4 ± 234.17	1106.20 ± 142.70	1126.8 ± 163.24	100–1300	10^3 /µL
	WBC	11.07 ± 1.71	12.20 ± 1.54	11.11 ± 2.27	9.86 ± 0.87	11.20 ± 1.99	12.23 ± 2.27	6– 17	10^3 /µL
28/42[a]	RBC	7.95 ± 0.35	7.768 ± 0.35	7.47 ± 0.16*	7.22 ± 1.20	7.61 ± 0.52	7.66 ± 0.60	7 – 10	10^6 /µL
	Hb	14.40 ± 0.65	14.12 ± 0.48	13.92 ± 0.55	13.12 ± 2.14	13.46 ± 0.68	13.66 ± 1.09	11 – 18	g/dL
	HCT	44.00 ± 2.13	43.12 ± 1.82	42.22 ± 1.55	39.80 ± 6.88	41.12 ± 1.79	41.88 ± 3.02	36 – 48	%
	MCV	55.28 ± 0.32	55.50 ± 0.93	56.60 ± 1.62	55.14 ± 0.23	54.06 ± 2.02	54.66 ± 1.05	53–59.5	fL
	MCH	18.10 ± 0.38	18.18 ± 0.46	18.64 ± 0.58	18.18 ± 0.43	17.66 ± 0.42	17.82 ± 0.13	18.3– 20	Pg
	MCHC	32.76 ± 0.75	32.78 ± 0.43	32.94 ± 0.30	33.04 ± 0.65	32.72 ± 0.84	32.60 ± 0.66	32.7–35.7	g/dL
	Platelet	1076.60 ± 65.31	633.00 ± 274.60*	1000.00 ± 106.28	721.20 ± 494.46	928.50 ± 412.34	1026.60 ± 173.47	100–1300x10	10^3 /µL
	WBC	7.73 ± 1.90	6.49 ± 1.06	6.78 ± 1.41	6.95 ± 3.32	6.30 ± 1.13	6.17 ± 3.49	6–17	10^3 /µL

([a]): 28 days for treatment groups and 42 days for satellite groups. RBC: Red blood cells, Hb: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, WBC: White blood cells. Group 1: water, Group 2: 200+100 mg/kg bw of *C. cajan* and *Z. officinale*, Group 3: 400+200 mg/kg bw of *C. cajan* and *Z. officinale*, Group 4: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*, Group 5: water, Group 6: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*. Data are represented as mean ± standard deviation (SD) (n=5). *p<0.05 Vs. Group 1 in treatment groups with Independent t-test.

Table 5: Blood biochemistry of male rats after the combined extract of *Cajanus cajan* and *Zingiber officinale* treatments

Days	Parameters	Treatment Groups				Satellite Groups		Normal Range	Unit
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6		
0	ALT	77.40 ± 12.66	71.60 ± 10.73	75.80 ± 13.53	91.20 ± 15.35	71.60 ± 15.54	78.20 ± 24.25	22 – 165	IU/L
	AST	105.80 ± 14.32	114.00 ± 11.33	104.80 ± 9.09	107.80 ± 11.25	100.60 ± 11.14	108.00 ± 23.11	16 – 414	IU/L
	Creatinine	0.72 ± 0.03	0.83 ± 0.07*	0.770.04	0.78 ± 0.09	0.70 ± 0.03	0.78 ± 0.09	0.2 – 0.8	mg/dL
	Urea	46.92 ± 5.41	49.74 ± 7.34	47.62 ± 8.82	51.86 ± 12.04	42.28 ± 7.36	55.96 ± 13.39	45 – 56.9	mg/dL
	TC	71.80 ± 18.75	62.00 ± 13.28	62.80 ± 7.19	73.80 ± 7.82	76.00 ± 8.51	84.60 ± 17.47	40 – 130	mg/dL
	Triglyceride	40.20 ± 4.20	32.80 ± 14.61	39.40 ± 15.53	39.00 ± 11.68	43.00 ± 8.39	41.60 ± 1.67	20 – 114	mg/dL
28/42[a]	ALT	50.00 ± 7.48	71.60 ± 10.28**	56.40 ± 5.89	62.80 ± 11.38	61.40 ± 9.42	64.00 ± 11.97	22 – 165	IU/L
	AST	150.00 ± 9.05	189.80 ± 57.21	86.40 ± 4.21**	107.20 ± 27.99*	111.00 ± 8.91	131.20 ± 22.55	16 – 414	IU/L
	Creatinine	0.75 ± 0.06	0.74 ± 0.05	0.72 ± 0.05	0.70 ± 0.06	0.91 ± 0.08	0.88 ± 0.05	0.2 – 0.8	mg/dL
	Urea	35.00 ± 4.79	42.00 ± 1.87*	36.80 ± 2.77	30.80 ± 3.96	43.40 ± 3.78	39.20 ± 2.28	45 – 56.9	mg/dL
	TC	59.40 ± 3.50	57.20 ± 7.19	60.20 ± 4.60	58.00 ± 7.71	41.20 ± 12.02	46.80 ± 21.92	40 – 130	mg/dL
	Triglyceride	39.60 ± 8.64	39.80 ± 11.84	56.80 ± 15.05	46.60 ± 19.48	74.40 ± 7.19	64.80 ± 2.28†	20 – 114	mg/dL

([a]): 28 days for treatment groups and 42 days for satellite groups. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TC: Total cholesterol. Group 1: water, Group 2: 200+100 mg/kg bw of *C. cajan* and *Z. officinale*, Group 3: 400+200 mg/kg bw of *C. cajan* and *Z. officinale*, Group 4: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*, Group 5: water, Group 6: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*. Data are represented as mean ± standard deviation (SD) (n=5). *p<0.05; **p<0.01 Vs. Group 1 in treatment groups with Independent t-test. †p<0.05 Vs. Group 6 in satellite group with Mann-Whitney test.

Biochemical parameters play a crucial role as indicators in toxicological assessment due to their ability to reflect the clinical signs and symptoms caused by toxic substances.[17] The liver and kidney are vital organs to evaluate the toxic effect of extract and drugs because both play an essential role in metabolism and vital function.[22] The liver produces alanine aminotransferase (ALT), which can be used as a sensitive indicator of liver injury. This enzyme will be released into the bloodstream when cells are damaged.[17] Likewise, the liver also produces aspartate aminotransferase (AST). AST is not specific to the liver but is also found in red blood cells, cardiac and skeletal muscles, and kidneys.[17] In the present study, male rats given with *C. cajan* and *Z. officinale* combination in 200+100 mg/kg bw showed a significant increase in ALT compared to the control treatment ($p<0.05$) (Table 5). Furthermore, a notable elevation in AST levels was observed in male rats administered with 400+200 and 800+400 mg/kg bw when compared to the control group ($p<0.05$; $p<0.01$). In contrast, there ware no difference in ALT and AST levels between female rats and the control group (Table 6). Nevertheless, these alterations were non-dependent doses and within the normal range in male rats of similar species, strains, and sex (ALT: 22 – 165 U/L, AST: 16 – 414 U/L).[20] The measurement of urea and creatinine levels is commonly used to assess renal function.[23] The creatinine levels reflect the glomerular filtration rate, whereas the urea levels indicate the capacity of the kidneys to excrete waste products.[16] Creatinine is naturally produced in the body and consistently released into body fluids steadily. Its concentration in the bloodstream is primarily regulated through glomerular filtration.[17] So, a quantitative measurement of creatinine in the blood can indicate impaired function and be indicative of many health problems.[24] Urea was overly produced in acute or chronic renal diseases. The decline in kidney function reduces urea clearance, causing urea to accumulate within the body.[25]

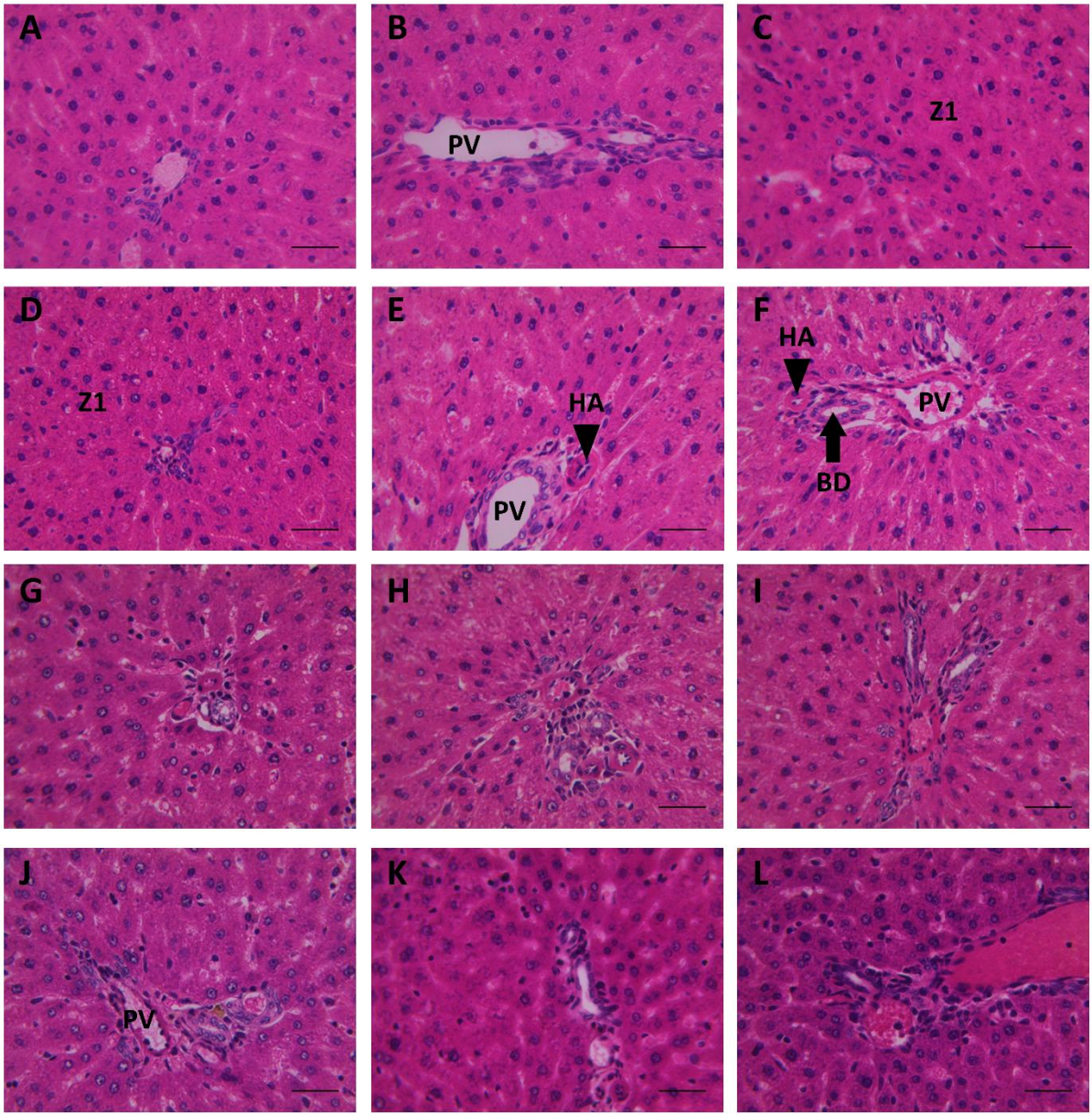

Figure 1: Photomicrograph of rat's liver treated with *C. cajan* and *Z. officinale.* A & G : Water, B & H: 200+100 mg/kg, C & I : 400+200 mg/kg , D & J: 800+400 mg/kg bw, E & K: Water , F & L: 800+400 mg/kg bw. A-F: Male rats, G-H: Female rats. A-D & G-J: 28 days for treatment groups, E-F & K-L: 42 days for satellite groups. Bar=50 µm. PV: Portal vein, HA: Hepatic artery, BD: Bile duct, Z1: Zone 1 (periportal).

Table 6: Blood biochemistry of female rats after the combined extract of *Cajanus cajan* and *Zingiber officinale* treatments

Days	Parameters	Treatment Groups				Satellite Groups		Unit	Normal Range
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6		
0	ALT	72.20 ± 14.07	71.80 ± 6.90	68.40 ± 14.36	60.20 ± 7.19	71.60 ± 18.67	70.60 ± 12.77	71– 201	IU/L
	AST	101.80 ± 17.45	108.60 ± 11.43	103.80 ± 19.61	93.80 ± 9.47	101.00 ± 13.69	110.80 ± 26.62	97 – 309	IU/L
	Creatinine	1.11 ± 0.53	0.81 ± 0.10	0.74 ± 0.12	1.13 ± 0.56	0.80 ± 0.05	0.69 ± 0.06†	0.2 – 0.8	mg/dL
	Urea	45.35 ± 8.23	53.82 ± 4.47	45.8 ± 7.87	45.80 ± 6.09	48.70 ± 6.30	40.46 ± 7.91	39 – 62	mg/dL
	TC	n.m	n.m	n.m	n.m	n.m	n.m	40 – 130	mg/dL
	Trigliserida	n.m	n.m	n.m	n.m	n.m	n.m	17 - 47	mg/dL
28/42[a]	ALT	92.40 ± 27.34	73.80 ± 7.79	81.80 ± 19.29	75.60 ± 14.41	77.20 ± 7.56	80.80 ± 21.45	25 – 36	IU/L
	AST	134.40 ± 35.78	119.00 ± 13.82	123.20 ± 12.31	139.80 ± 79.96	117.20 ± 21.22	116.00 ± 18.43	85 – 123	IU/L
	Creatinine	0.71 ± 0.04	0.85 ± 0.01**	0.79 ± 0.05*	0.73 ± 0.04	0.81 ± 0.03	0.79 ± 0.02	0.2 – 0.8	mg/dL
	Urea	53.76 ± 10.98	0 ± 4.8153.8	45.78 ± 6.33	40.68 ± 6.17	49.44 ± 6.49	42.82 ± 1.53	39 – 62	mg/dL
	TC	n.m	n.m	n.m	n.m	47.00 ± 11.89	50.80 ± 7.19	40 – 130	mg/dL
	Triglyceride	n.m	n.m	n.m	n.m	37.40 ± 9.23	42.00 ± 16.15	17 - 47	mg/dL

([a]): 28 days for treatment groups and 42 days for satellite groups. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TC: Total cholesterol. Group 1: water, Group 2: 200+100 mg/kg bw of *C. cajan* and *Z. officinale*, Group 3: 400+200 mg/kg bw of *C. cajan* and *Z. officinale*, Group 4: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*, Group 5: water, Group 6: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*. Data are represented as mean ± standard deviation (SD) (n=5). *p<0.05; **p<0.01 Vs. Group 1 in treatment groups with Independent t-test. †p<0.05 Vs. Group 5 in satellite group with Mann-Whitney test. n.m: not measured.

Table 7: Histopathology scoring of organ rats after combined extract of *Cajanus cajan* and *Zingiber officinale* treatments

	Groups	Scoring Value								
		Brain	Lungs	Heart	Liver	Kidney	Spleen	Pancreas	Testis	Ovary
	Treatment Groups									
♂	Group 1	0	0	0	0	0	0	0	0	0
	Group 2	0	0	0	0	0	0	0	0	0
	Group 3	0	0	0	0	0	0	0	0	0
	Group 4	0	0	0	0	0	0	0	0	0
♀	Group 1	0	0	0	0	0	0	0	0	0
	Group 2	0	0	0	0	0	0	0	0	0
	Group 3	0	0	0	0	0	0	0	0	0
	Group 4	0	0	0	0	0	0	0	0	0

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♂	Group 5	0	0	0	0	0	0	0	0	0
	Group 6	0	0	0	0	0	0	0	0	0
♀	Group 5	0	0	0	0	0	0	0	0	0
	Group 6	0	0	0	0	0	0	0	0	0

n=3; 0: no abnormalities. Group 1: water, Group 2: 200+100 mg/kg bw of *C. cajan* and *Z. officinale*, Group 3: 400+200 mg/kg bw of *C. cajan* and *Z. officinale*, Group 4: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*, Group 5: water, Group 6: 800+400 mg/kg bw of *C. cajan* and *Z. officinale*.

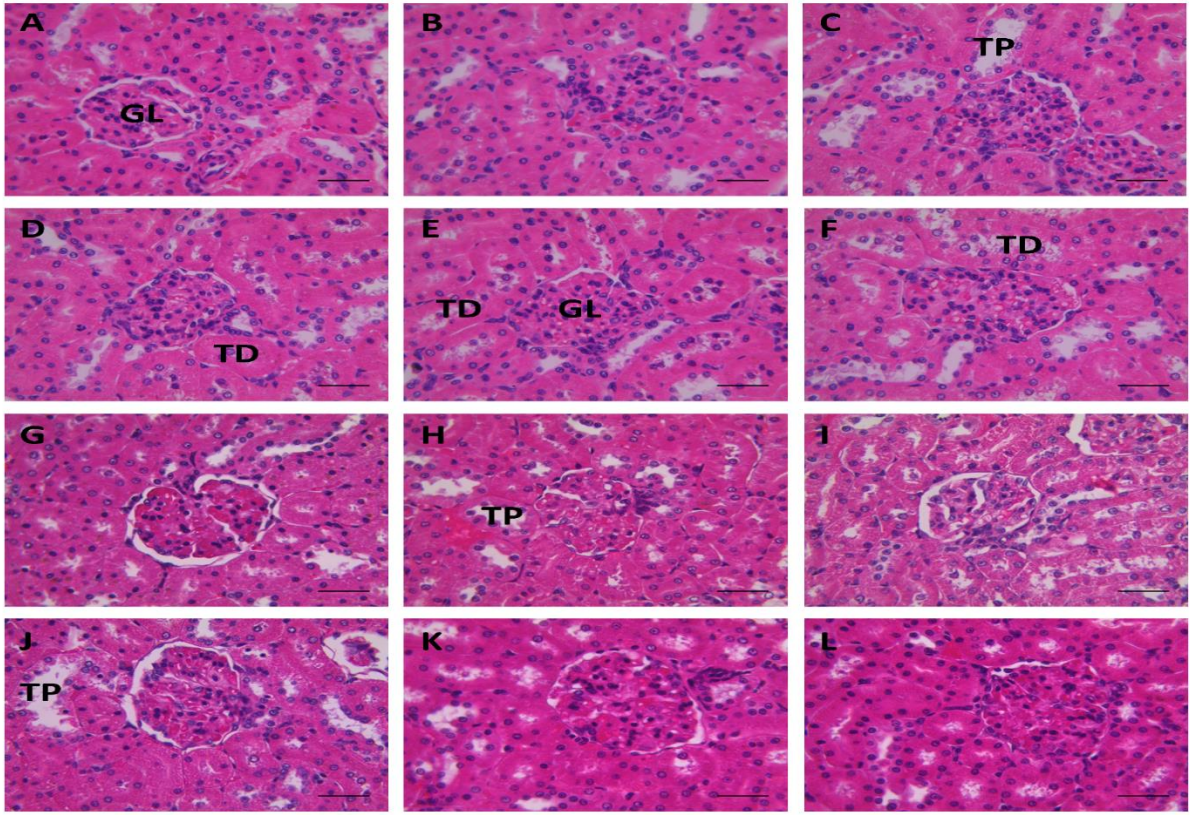

Figure 2: Photomicrograph of rat's kidney treated with *C. cajan* and *Z. officinale*. A & G : Water, B & H: 200+100 mg/kg, C & I : 400+200 mg/kg , D & J: 800+400 mg/kg bw, E & K: Water , F & L: 800+400 mg/kg bw. A-F: Male rats, G-H: Female rats. A-D & G-J: 28 days for treatment groups, E-F & K-L: 42 days for satellite groups. Bar=50 µm. GL: Glomerulus, TP: Proximal tubule, TD: Distal tubule.

The present study found that male rats treated with both extracts significantly increased urea in group 2 at 28-day treatment periods compared to group 1 ($p<0.05$) (Table 5). In addition, after 28-day treatment of both extracts, female rats showed a significant increase in creatinine in 200+100 and 400+200 mg/kg bw ($p<0.01$, $p<0.05$, respectively) compared with control. At 42 days, there was no significantly different in urea and creatinine between groups 5 and 6 in satellite group. Although the creatinine and urea changed, it was within the normal range in similar species, strains, and sex (creatinine: 0.2-0.8 mg/dl, urea: 45-59 mg/dl).[20]

Total cholesterol and triglycerides in the repeated dose 28-day oral toxicity study are observed to evaluate the impact of toxic substances on lipid metabolism. High total cholesterol and triglyceride levels may indicate lipid dysregulation. There was no significant difference in male rats in all treatment groups (Table 5). In satellite groups, triglyceride levels in group 6 were significantly lower than group 5 ($p<0.05$). However, this change was within the normal range (20-114 mg/dl).

Effect on Histopathology of Organs

Table 7 show the histopathology scoring of organ of rats treated with the combined extract of *C. cajan* and *Z. officinale*. In all the groups, there were no detected remarkable pathological changes in all organs, including the liver (Figure 1), kidney (Figure 2), brain (Figure S1), lungs (Figure S1), heart (Figure S3), spleen (Figure S4), pancreas (Figure S5), testis (Figure S6), and ovary (Figure S6). No abnormalities were found in the histopathological observation of male and female rats. This study concluded that the administration of the combined extract of *C. cajan* and *Z. officinale* for 28 days did not produce structural alterations in all organs.

There were no abnormalities in the liver, such as degeneration and necrosis in liver zonation (Zone 1, 2 and 3) (Table 7 and Figure 1). Previous research revealed that Zone 2 hepatocytes play a crucial role in maintaining a balanced state of cell growth. In contrast, hepatocytes in zones 1 and 3 can regenerate the liver following damage specifically to zones 3 and 1, respectively.[26] In repeated dose 28-day oral toxicity study, no abnormalities were found in liver rats treated with *C. cajan* extract at doses of 1.5, 3, and 6 mg/kg bw.[10] Lipid deposition was also not found in the liver (Figure 2). So, these findings supported that the significant changes in ALT, AST and triglyceride were considered biologically insignificant and incidental (Table 5).[21] The liver plays regulation and metabolism of total cholesterol and triglycerides. The high total cholesterol and triglycerides increased lipid deposition in the hepatic cytoplasm.[27]

No abnormalities were found in the kidney in histopathological observation, including degeneration and necrosis in proximal tubules and mesangial cells (Table 7 and Figure 2). In previous research, there was no toxic effect in the kidney of rats after 28-day treatment periods of *C. cajan* extract.[10] Furthermore, these findings supported that the significant changes in some of the biochemistry parameters were considered biologically insignificant and incidental.[21]

Conclusion

The repeated dose 28-day oral toxicity study revealed that oral administration of the combination of *Cajanus cajan* and *Zingiber officinale* for 28-day treatment did not exhibit any toxic effects on male and female rats. No cumulative effects of the combined extracts were observed after 14-day recovery periods. This fact was evidenced by insignificant changes in body weight, hematology, biochemistry, and histopathological observations. The present study did not identify the existence of no observed adverse effect level (NOEL) in rats given with the combination of *C. cajan* and *Z. officinale* extracts at 200+100, 400+200, and 800+400 mg/kg bw.

Conflict of Interest

The authors declare no conflict of 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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