Acute and Sub-chronic Toxicity Studies of Combination of *Physalis angulata L.* (*Cecendet*) Extract and Methylprednisolone on Animals

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Abstract

Objective: To evaluate the safety of combination of *Physalis angulata L.* (*Cecendet*) extract and methylprednisolone through a single dose in mice (acute toxicity) and repeated doses within 90 days in rats (subchronic toxicity).

Methods: : Acute toxicity test was conducted to 2 groups of female mice by monitoring their behavior and death at 4 hours and 24 hours after *cecendet* adminstration. While subchronic toxicity test was conducted to 6 groups of rats as normal group, control group, and receive varoius *cecendet* dose. Each group was combined with methylprednisolone 5 mg/kg b.w. at first month and gradually taperred to 3.2 mg/kg b.w. at third month. Subchronic toxicity was evaluated by monitoring rats' behavior, mortality, blood biochemistry, blood cell count, urinalysis, and organ macroscopic and histologic.

Results: The study showed *cecendet* extracts did not affect mice behavior in single dose therapy up to 5 g/kg b.w. and had LD50 of more than 5 g/kg b.w., which was categorized as practically non-toxic. The sub-chronic toxicity study showed that up to 1 g/kg b.w. of *cecendet* extracts that was administered for 90 days did not cause death, was not organ toxic, did not affect blood biochemistries, blood cell count, and urinalysis.

Conclusions: *Cecendet* extract and methylprednisolone combination was safe based on acute and sub-chronic toxicity data.

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Introduction

Systemic Lupus Erythematosus (SLE) is regarded as a chronic inflammatory disease with unknown etiology. The SLE has various clinical manifestations, natural history of disease, and prognosis. The disease primarily affects women of childbearing age with high mortality rate.[1] Corticosteroid is one of the major drug therapies for SLE as anti-inflammatory and immunosuppressant. One potential and recommended corticosteroid in Indonesia for SLE patient is methylprednisolone with various dosages, depending on the severity of the disease. The recommended low-dose corticosteroid for SLE maintenance therapy is less than or equal to 7.5 mg of prednisone and gradually tapers off over several weeks to prevent withdrawal effect until the steroid can be stopped. Thus, the dose should be reduced by 20–25% every week or longer.[2]

Physalis angulata L., known as *cecendet*, is a herb with several characteristics such as 0.1–1 m of height, upright, and with strong branch. In Java Island, Indonesia, it grows on lowland up to 1,200 m above the sea level. *Cecendet* has been used by society through generations as antidiabetic, antibacterial, antiviral, anti-inflammatory, antioxidant, analgesic, diuretic, cough reliever, and antitumor.[3] The laboratory studies proven that cecendet suppress immune system by decreasing T cell proliferation, lymphocytes function, and

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effects on macrophage activation.[4,5] *Cecendet* has anti lupus effect to pristane-induced lupus animal model such as reducing proteinuria level and anti-inflammatory effect.[4–8]

Based on the previous studies, *cecendet* has potential effect for supplementary therapy in lupus patients. Therefore, its safety profile must be investigated through acute and sub-chronic toxicity studies. The preliminary study for acute toxicity showed that the cecendet alone has an LD50 of more than 5 g/kg b.w. and is categorized as practically non-toxic. Sub-chronic toxicity of *cecendet* alone to rat did not affect blood biochemistry (glucose, total cholesterol, triglycerides, SGPT, SGOT, creatinine, and urea); index of vital organs such as the liver, kidneys, and heart are not different from control; urinalysis parameter were not affected; and histology of the kidneys, liver, heart, spleen and lungs showed no abnormalities compare to control group.

Most SLE patients need immunosuppressive agents, therefore toxicity study of cecendet extract and corticosteroid combination is needed. Therefore, this study evaluated the safety of combination of *Cecendet* extract and methylprednisolone through a single dose in mice with acute toxicity and repeated doses within 90 days in rats with subchronic toxicity.

Methods

The material used was cecendet extract in amylum maydis (5:4 ratio). The extract was prepared by PT. Phytochemindo Reksa, a pharmaceutical manufacturing certified for good manufacturing practice for traditional medicine (Certificate Number: 0104/CPOTB/14.1/43/VIII/2008) by National Agency for Drug dan Food Control of The Republic of Indonesia. Methylprednisolone were obtained from marketplace as a generic brand of 4 mg tablet.

Female mice weighing 29–34 grams for acute toxicity study were obtained from Animal Laboratory, School of Pharmacy, Bandung Institute of Technology; while male

Table 1 Rats Organ Index after Cecendet Extract and Methyprednisolon Administration for 90 days

Group	Control	225 mg/ kg b.w.	900 mg/ kg b.w.	1800 mg/ kg b.w	Satellite Control	Satellite 1800 mg/ kg b.w.
Male						
Liver	2.86±0.47	2.88±0.36	2.82±0.18	2.77±0.17	2.71±0.36	2.73±0.17
Lungs	0.61±0.10	0.77±0.37	0.66±0.10	0.62±0.13	0.59±0.13	0.71±0.35
Heart	0.31±0.05	0.36±0.03	0.37±0.04	0.37±0.06	0.33±0.04	0.40±0.06
Spleen	0.19±0.019	0.19±0.025	0.20±0.027	0.18±0.028	0.20±0.025	0.19±0.052
Kidney	0.59±0.10	0.62±0.05	0.62±0.05	0.65±0.04	0.56±0.05	0.64±0.06
Adrenal glands	0.019±0.003	0.016±0.004	0.018±0.004	0.016±0.002	0.020±0.005	0.019±0.005
Testis	0.91±0.13	1.00±0.07	0.98±0.12	0.99±0.09	0.93±0.16	0.99±0.23
Seminal vesicles	0.50±0.08	0.43±0.06	0.42±0.08	0.51±0.11	0.48±0.13	0.47±0.11
Female						
Liver	3.00±0.22	3.22±0.21	3.17±0.35	3.02±0.35	2.95±0.49	2.97±0.36
Lungs	0.60±0.06	0.71±0.08	0.75±0.12	0.80±0.27	0.65±0.11	0.80±0.24
Heart	0.37±0.03	0.36±0.02	0.39±0.03	0.36±0.03	0.36±0.04	0.43±0.06
Spleen	0.20±0.024	0.21±0.038	0.23±0.045	0.24±0.051	0.20±0.033	0.24±0.034
Kidney	0.60±0.08	0.62±0.05	0.61±0.05	0.64±0.06	0.63±0.08	0.64±0.06
Adrenal glands	0.027±0.007	0.033±0.007	0.032±0.006	0.031±0.007	0.033±0.007	0.040±0.006
Ovarium	0.055±0.007	0.053±0.007	0.050±0.012	0.053±0.011	0.058±0.011	0.059±0.007
Uterus	0.32±0.16	0.23±0.03	0.32±0.28	0.29±0.13	0.28±0.11	0.25±0.06

and female Wistar rats weighing 200–210 grams for sub-chronic toxicity study were obtained from D'Wistar Laboratory Animal Suppliers, Bandung.

The procedure of this study was carried out based on guidelines for invivo nonclinic toxicity from National Agency for Drug dan Food Control (NADFC) of The Republic of Indonesia. Acute toxicity test according to the fixed dose method of acute oral toxicity, while subchronic test according to the 90 days of oral subchronic toxicity test. All the procedures were approved by The health research ethic committee Faculty of Medicine Universitas Padjadjaran Bandung with ethical approval no 262/UN6.C1.3.2/KEPK/PN/2061.

Ten female mice were acclimatized for 7 days prior to the study and fasted for 4 hours prior to test substance administration, while water was still provided. Mice were randomly divided into 2 groups (each group consist of 5 mice): a dose group and a control group.[9]

Cecendet dose of 5 g/kg b.w. (5000 mg/kg b.w.) was combined with methylprednisolone dose of 0.14 mg/20 g b.w. or 7.25 mg/kg b.w, which was converted from human dose of 56 mg/70 kg. Mice received test preparation solution for 1 mL/20 g b.w. orally. They were also given methylprednisolone solution 0.5 mL/20 g b.w. 5 minutes afterwards.

In the previous study, a limit test at one dose level of 5000 mg/kg did not show mortality and based on the guide line of NADFC further testing at the next lower level did not need to be carried out, therefore this dose was used in the combination test with methylprednisolon.

Both animal behavior and death were observed for 4 hours and then 24 hours after

Table 2 Hematology Profile after Test Preparation Administration for 90 Days

Group	Control	225 mg/ kg b.w.	900 mg/ kg b.w.	1800 mg/ kg b.w	Satellite Control	Satellite 1800 mg/ kg b.w.
Male						
Hematocrit (%)	34.0±6.4	32.5±4.0	31.6±3.2	33.8±6.0	32.2±4.1	30.8±2.7
Hemoglobin (g/dl)	13.5±0.9	13.1±1.0	13.3±0.8	13.1±1.6	13.3±1.2	13.3±1.2
Σ Thrombocyte (10^5/mm^3)	1406.1± 276.9	1483.1± 194.6	1334.7± 153.0	1394.9± 163.0	1258.7± 92.6	1260.6± 325.8
Σ Leukocyte (103/mm^3)	8.0± 2.6	5.4±0.8*	4.9±1.2*	4.0±1.0*	6.6±1.4	3.5±0.5
Σ Eryhtrocyte (106/mm^3)	5.7±1.1	5.5±0.6	5.4±0.6	5.7±0.9	5.5±0.7	5.3±0.5
MCH (pg/cell)	49.0±6.7	48.4±4.4	50.0±3.5	46.0±4.9	49.3±4.8	49.8±3.8
MCHC (g/dL)	135.7±19.1	135.8±13.2	141.3±9.8	130.6±14.6	139.0±13.5	144.1±14.3
MCV (m3/cell)	36.1±1.1	35.7±0.5	35.4±0.7	35.3±0.9	35.5±0.5	34.7±1.8
Female						
Hematocrit (%)	32.0±4.1	38.8±9.1	34.9±2.9	33.6±4.0	37.2±5.8*	35.0±8.3
Hemoglobin (g/dL)	12.5±1.1	13.0±1.6	12.1±0.8	12.7±1.2	13.8±1.3	12.5±0.8
Σ Thrombocyte (10^5/mm^3)	1346.3± 186.8	1381.8± 298.0	1345.8± 297.8	1807.8± 582.5	1231.0± 133.6	1087.4± 163.8
Σ Leukocyte (103/mm^3)	5.3±1.7	7.3±1.7*	4.9±0.9	5.6±2.0	4.7±0.8	5.2±1.3
Σ Eryhtrocyte (106/mm^3)	5.7±1.1	6.4±1.5	5.9±0.5	5.7±0.7	6.3±1.0	5.9±1.4
MCH (pg/cell)	45.2±6.8	41.2±5.2	41.4±4.4	46.0±3.3	44.5±4.5	43.9±6.8
MCHC (g/dL)	131.2±13.6	114.0±15.0	116.7±13.0	127.1±9.0	125.2±12.7	122.3±19.1
MCV (m3/cell)	34.4±3.4	36.2±0.6	35.5±0.7	36.2±0.7	35.7±0.5	35.9±1.1

Note: * $p<0.05$ compared to control

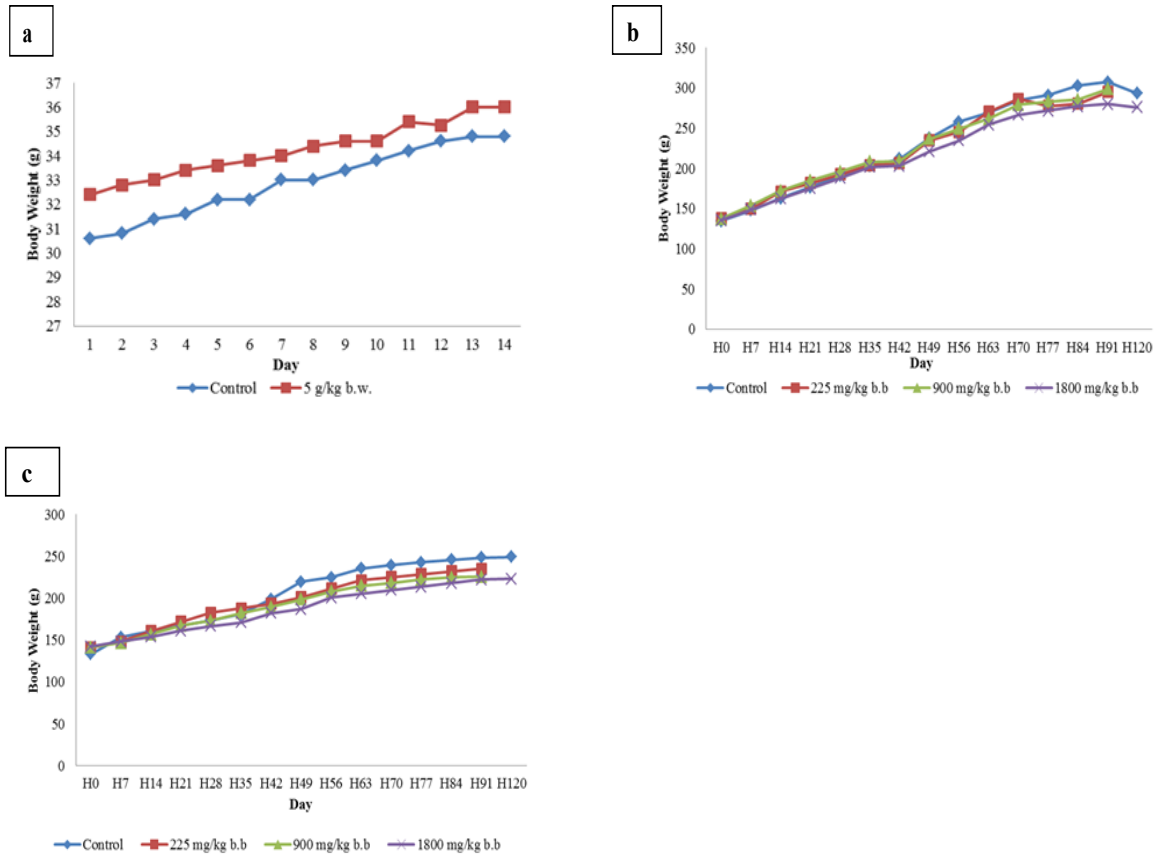

Fig. 1 Mice Body Weight for 14 Days after Administration of *Cecendet* Extract (a), Male Rat Body Weight Profile for 120 Days (b), Female Rat Body Weight Profile for 120 days (c)

administration. Body weight were measured daily for 14 days along with observation of toxicity signs. At day fourteenth, all mice were sacrificed, macroscopic presentation of organs such as liver, kidneys, spleen, adrenal glands, heart, lungs, ovaries, testis and gastric mucosal were observed.

The rats were acclimatized for 7 days prior to the study. Rats were randomly divided into six groups, each consist of 10 male rats and 10 female rats per group, consisting test, control and satellite group. Test group received 3 *Cecendet* doses of 225 mg/kg b.w., 900 mg/kg b.w., and 1800 mg/kg b.w. combined with methylprednisolone; and satellite group received cecendet dose of 1800 mg/kg b.w. combined with methylprednisolone. Methylprednisolone dose was tapered off consistently with the human dosage, for day 1st–30th was 5 mg/kg b.w, day 31st–60th was 4 mg/kg b.w, and day 61st-90th was 3.2 mg/kg b.w.

Behavior was observed prior to administration, on day 91st, and day 120th for satellite group. At the end of the experiment (test animal and control on day 91st; satellite group on day 120th) urinalysis was done by collecting urine approximately 16 hours to observe urine color, specific gravity, and pH.[9] Blood was collected from the tail to observe erythrocytes count, leucocytes count, hemoglobin, and hematocrit (volume of red blood cells compared to total blood volume). Blood chemistry (ALT, AST, and creatinine) examination was done by using spectrophotometer tool "Tecno 168" with human reactor.

All animals then were sacrificed to observe the organs (liver, spleen, kidneys, adrenal glands, heart, lungs, ovaries, testis and seminal vesicles) macroscopically. In this study, organ histology examination for liver, lungs, spleen, heart and kidneys were done to all groups.[9]

Table 3 Clinical Biochemistry Profile after Test Preparation Administration for 90 days

Group	Control	225 mg/ kg b.w.	900 mg/ kg b.w.	1800 mg/ kg b.w	Satellite Control	Satellite 1800 mg/ kg b.w.
Male						
ALT (U/L)	38.6±5.9	36.0±4.7	41.6±4.4	41.5±5.6	47.0±13.1	38.6±3.1
AST (U/L)	135.0±28.3	123.0±11.0	118.1±11.8	123.7±11.4	125.3±11.4	124.3±8.9
Creatinin (mg/dL)	0.45±0.09	0.42±0.15	0.38±0.16	0.47±0.15	0.42±0.13	0.35±0.12
Glucose (mg/dL)	112.8±25.0	142.0±17.8	130.3±31.8*	106.9±16.4*	120.3±15.8	156.5±19.8
Total cholestrol (mg/dL)	82.0±12.8	97.5±62.7	78.1±13.7	87.1±59.3*	88.4±31.4	91.7±12.9
Trigliseride (mg/dL)	147.7±23.7	110.1±37.6	109.6±42.7	122.1±13.8*	127.2±22.1	113.0±22.3
Urea (mg/dL)	27.9±2.8	29.2±5.6	38.7±3.7	38.6±9.0*	25.2±3.8	32.9±6.2
Female						
ALT (U/L)	34.1±5.5	36.1± 4.0	31.8±4.8	29.1±3.1	32.5±5.4	33.7±5.2
AST (U/L)	98.2±19.8	123.0±19.3	111.7±21.4	116.1±34.8	95.2±16.9	105.3±7.5
Creatinin (mg/dL)	0.34±0.15	0.31±0.20	0.40±0.26	0.30±0.17	0.23±0.09	0.29±0.12
Glucose (mg/dL)	169.3±32.6	161.6±51.2	159.6±41.3	119.3±24.3	179.9±24.5	161.1±17.8
Total cholestrol (mg/dL)	85.9±12.5	106.8±43.6	115.8±26.9	118.1±57.6	110.8±59.7	91.4±11.2
Trigliseride (mg/dL)	170.1±31.5	149.9±39.2	143.3±27.8	132.2±42.8*	187.6±74.9	149.0±55.3
Urea (mg/dL)	39.9±8.4	39.2±7.5	38.4±10.3	37.9±19.8	38.8±7.2	47.9±9.2

Note: * $p<0.05$ compared to control

Results

Animal observation at 4 hours and 24 hours after administration of 5 g/kg b.w. of *cecendet* extract combined with methylprednisolone on acute toxicity test show no behavioral difference of test group compare to control group. The animal behaviors such as hanging, retablishment, flexion response, and Haffner response were showed equal in both group and there were no dead animals was observed.

There was no sign of toxicity such as straub reaction, vocalization, writhing, convulsion, tremor, lacrimation, salivation, piloerection, and ptosis on obaservation for 14 days after administration. Body weight development profile for 14 days of control and test group were compared and described (Fig. 1a). It showed that body weight gain in acute toxicity study from both control and test group are similar.

All experimental animals of subchronis toxicity study were not affected by combination of *Cecendet* extracts and methylprednisolone, all parameters were normal compared to control group. There were no toxicity signs of straub reaction, vocalization, writhing, convultion, tremor, lacrimation, salivation, piloerection, and ptosis. Other behaviors such as hanging, retablishment were normally done, all rat group showed flexion response, Haffner response, pineal and corneal reflexes. While defecation, urination, and grooming were equal in both group.

Body weight development profile of subchronic toxicity study for male rat groups and female rat groups were described (Fig. 1b, c). The body weight increased in both male and female groups, and similar with control group.

Macroscopic observation on liver, heart, lung in both male and female rats showed no abnormalities in color and shape compared to control group. The ratio of organ to body weight after 90 days adminstration revealed that heart, liver, lung, spleen, kidney on male and female test group were not significantly different with control group (Table 1). Testis in male animals, ovaries and uterus in female

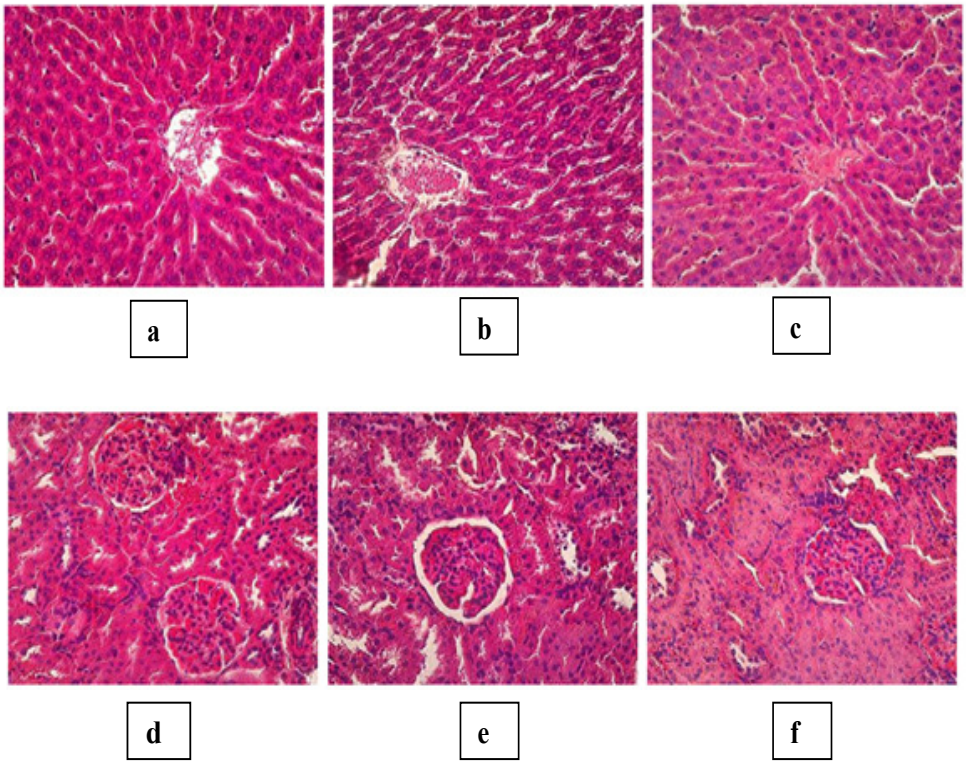

Fig. 2 Liver Histology in Female Rat after 90 Days of *Cecendet* Extract Administration (40x). (a) Control Group (b) Extract Dose of 225 mg/kg b.w. and Methylprednisolone (c) Extract Dose of 1800 mg/kg b.w. and Methylprednisolone. Kidney Histology in Female Rats after 90 days of *Cecendet* Extract Administration (40x). (d) Control Group (e) Extract Dose of 225 mg/kg b.w. and Methylprednisolone (f) Extract Dose of 1800 mg/kg b.w. and Methylprednisolone

animals were similar compared to control groups.

Blood parameter showed that there was no difference between test group and control group on both male and female groups (Table 2). Statistycal analysis with one way ANOVA showed significance decerased of leukocytes number in male rat was in all doses ($p<0.05$).

Blood chemistries to examine combination of *cecendet* extract and methylprednisolone effects on heart, liver and kidneys were presented (Table 3).[10,11] The ALT and AST in all dose groups did not show any differences compared to control group. Similarly, blood creatinine level did not significantly differ between test and control group, both in male and female.

There were significant changes in blood glucose level in comparison with control group, such as an increase blood glucose level in male group with *cecendet* dose of 900 mg/kg b.w. and satellite group, while a decrease blood glucose level in female group with *cecendet* dose of 1800mg/kg b.w. and satellite, but all of glucose level were within normal limit (80–300mg/dL). In group with *cecendet* dose of 225 mg/kg b.w., there was no difference in total cholesterol level compared to control group. Nevertheless, in group with dose of 900 and 1800 mg/kg b.w., there were slight increase in total cholesterol level. Triglycerides level decreased in both group with cecendet dose of 1800 mg/kg b.w. and satellite male group. Urea level increased only in male group with cecendet dose of 900 and 1800 mg/kg b.w. From the results, group with cecendet dose of 225 mg/kg b.w. did not affect rats' blood biochemistries.

Table 4 Urinalysis Profile after Test Preparation Administration for 90 days

Group	Control	225 mg/ kg b.w.	900 mg/ kg b.w.	1800 mg/ kg b.w	Satellite Control	Satellite 1800 mg/ kg b.w.
Male						
Spesific gravity (g/mL)	1.19± 0.13	1.16± 0.02	1.05±0.08	1.11±0.01	1.17±0.04	1.12±0.11
pH	8.0±1.0	7.6±1.5	6.4±0.6	6.6±0.6	6.6±0.9	6.8±0.8
Female						
Spesific gravity (g/mL)	1.13±0.05	1.07± 0.01	1.05±0.06	1.08±0.03	1.21±0.04	1.16±0.07
pH	5.8±0.8	8.4±1.1	7.0±1.2	7.4±1.1	6.6±2.1	7.4±1.1

Urinalysis parameter in this study, specific gravity and urine pH level showed no significant changes both in test and control group, therefore the test preparation was non-toxic to kidney (Table 4). In liver and kidney observations, no differences were found microscopically both in test and control group. Normal hepatocytes in liver histology, both in test and control groups, Kuppfer cells were well distributed in female and male rats (Fig. 2a–c). Kidney histology showed normal glomerulus and bowman's capsule in male and female rats both in test and control group (Fig. 2d–f).

Discussion

The main hindrance to the use of natural or herbal as a supplement therapy for an illness is the lack of scientific data regarding its efficacy and safety. On the other hand, the use of traditional herbs has long been proven to have efficacy for several diseases. So, scientific research is needed to prove the safety of its herb, particularly those indicated to reduce the symptoms of a chronic disease. *Cecendet* is a herb that has long been used as an anti-inflammatory in rheumatic diseases and several previous studies have indicated that this cetendet has anti-inflammatory effect in the lupus animal model.[3,6] In current study, acute and sub-chronic toxicity studies of *cecendet* combined with methylprednisolone were carried out, as a condition adapted to lupus patients who were taking methylprednisolone as the main drug for lupus. The result of an acute toxicity test of 5 g/kg b.w. *cecendet* extract combined with methylprednisolone showed no sign of toxicity and no animal dead. Therefore, the *cecendet* extract has a lethal dose 50 (LD50) higher than 5 g/kg b.w. and according to Hodge and Sterner toxicity scale is categorized practically non-toxic.[9]

Body weight development profile in acute and sub-chronic toxicity studies showed body weight increasing of all test animals, indicates that cecendet extract does not have general toxic effects and not reducing appetite.[12] The liver plays key role in many metabolic process of the body, its inflammation and necrotic are identified by increasing of serum Alanine Aminotransferas (ALT) and Aspartate Aminotransferas (AST) level. In this study, the results of ALT and AST examination in all group of test animals did not show significant differences with control group, indicates that cecendet extract is not toxic to the liver.[10,12]

Renal function test should be performed on blood sample to investigate major toxic affect of tested material to kidney by monitoring creatinine and urea level.[11] The result of creatinine and urea level of all animal groups in this study were not difference compare to control group, it is indicate that cecendet extract is not affect the kidney function.

In addition, in current study show the significance decerased of leukocytes number in male rat of all group compare to control group. Generally, leukocytes is often associated with infection, as leukocytes number increase due to infection. Antimicrobial effects of cecendet may have caused decrease in leukocytes number.

In conclussion, this study demonstrated that administration of *cecendet* extract in

combination with methylprednisolone was safe both as single dose or repeated dose, based on acute and sub-chronic toxicity evaluation to mice and rats. Cecendet extracts with methylprednisolone had LD50 more than 5 g/kg b.w. and is categorized as practically non-toxic.

Body weight profile, behavior, blood biochemistry, blood, urinalysis parameter, and body organs (both macroscopically and microscopically) were not affected by cecendet (225 mg/kg b.w.) in combination with methylprednisolone (5mg/kg b.w. for the first month, 4 mg/kg b.w. for the second month, and 3.2 mg/kg b.w. for the third month).

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