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Original Article

Evaluation of acute and sub-acute toxicity of *Curcuma aeruginosa* Roxb. essential oil in Sprague Dawley rats

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Abstract

Background and purpose: *Curcuma aeruginosa* rhizome essential oil (CREO) is widely used in traditional medicine owing to its diverse biological activities. However, no information regarding its potential toxicity is available. This study aimed to evaluate the potential acute and sub-acute oral toxicities of CREO in Sprague Dawley rats.

Experimental approach: CREO was isolated *via* steam distillation and characterized using GC/MS. For acute toxicity, rats were divided into four groups and administered CREO at 2, 4, 8, and 16 g/kg. For the sub-acute evaluation, 30 male and 30 female rats were divided into 6 groups (1 control, 3 treatment doses, and 2 satellite), with doses of 50, 100, and 200 mg/kg BW administered for 28 days.

Findings/Results: GC/MS analysis identified eucalyptol, camphor, and epicurzerenone as the main phytochemically active components in CREO. The acute toxicity test demonstrated that CREO was toxic only at very high doses, with a lethal dose (LD_{50}) of 5662 mg/kg of body weight. Evaluation of sub-acute toxicity showed no significant changes in body weight, hematological, biochemical, and histopathological parameters in rats receiving CREO at doses < 200 mg/kg. However, rats that received CREO at 200 mg/kg showed liver early abnormalities. Similar to most natural extracts, CREO showed a hormetic dose response.

Conclusion and implications: This study suggests that CREO can be safely administered orally for therapeutic purposes at controlled doses. However, prolonged consumption and/or high doses may pose potential risks. Further evaluations are required to determine possible long-term effects.

Keywords: *Curcuma aeruginosa*; Essential oil; Histopathology; Rhizome; Toxicity.

INTRODUCTION

Over the past few years, essential oils and their derivatives have gained attention because of their tolerability and effectiveness in the prevention and treatment of various diseases, including cancer, metabolic syndromes, and oral diseases (1-4).

Many essential oils derived from clove, oregano, thyme, nutmeg, basil, mustard, and cinnamon have been recognized as safe by the United States Food and Drug Administration (FDA) (5,6). However, excessive ingestion of some of these essential oils can cause adverse effects, including headache, nausea, and hypoxia (1,7). Some essential oils can trigger neurotoxicity as well as hepatic and kidney damage (8,9). Therefore, evaluating the toxicological effects of essential oils is crucial to validate their safe prophylactic and therapeutic use (3).

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Curcuma aeruginosa Roxb. is native to tropical regions. In Indonesian, it is called temu hitam or temu ireng; in Thai, Wan MaHa-Mek; and in English, pink and blue ginger. It belongs to the Zingiberaceae family and contains essential oils with distinctive aromatic profiles, mainly in the rhizomes and leaves (10). *C. aeruginosa* and its rhizome essential oils have several bioactive properties, including antioxidant (11), antimicrobial (12), antibiofilm (13,14), and anti-inflammatory properties (15).

C. aeruginosa rhizome essential oil (CREO) contains secondary metabolites of the terpenoid group, particularly monoterpenes and sesquiterpenes (12). The major compounds found in CREO include camphor (29.39%) and germacrone (21.21%) (12). However, other studies have reported that the main components in CREO include curzerenone (24.6%), 1,8-cineole (11.0%), camphor (10.6%), zedoarol (6.3%), isocurcumenol (5.8%), curcumenol (5.6%), and furanogermenone (5.5%) (16).

To the best of our knowledge, no studies have investigated the oral toxicity of CREO. Evaluation of the oral toxicity of CREO is essential to validate its safety in use, as drug and chemical safety are assessed using animal models, especially rodents. Preclinical toxicological testing is commonly performed in rats, as it usually shows good safety translation for the clinical stage in humans (17,18). This study aimed to investigate the acute and sub-acute oral toxicity of CREO in Sprague Dawley (SD) rats and characterize its phytochemical content by gas chromatography/mass spectrometry (GC/MS).

MATERIALS AND METHODS

Plant collection

Fresh rhizomes of *C. aeruginosa* Roxb. were obtained from the Tropical Biopharmaca Research Center Conservation and Cultivation Station (BCCS), Bogor, Indonesia. Two botanists, Taopik Ridwan (BCCS) and Dr. Joeni Setijo Rahajoe (Research Center for Biology, LIPI), identified the plant. Voucher specimens of *C. aeruginosa* were collected at the BCCS (collection number BMK0097082016).

Distillation and phytochemical content analysis

The rhizomes of *C. aeruginosa* were cleaned using fresh water and sliced crosswise at a 2-3 mm thickness. The essential oil was obtained from steam distillation of sliced rhizomes at 80-100 °C for 6 h. Subsequently, CREO was separated from hydrosol, and Na_2SO_4 was added to absorb the water. The phytochemical content of CREO was analyzed by GC/MS using a GCMS-QP2020 NX (Shimadzu Corporation, Japan) equipped with a quadrupole mass analyzer and an electron multiplier detector. A Rxi-5Sil MS Shimadzu capillary column (fused silica column incorporated with phenyl groups in the polymer backbone, 30 m × 0.25 mm i.d., 0.25 µm d.f.) was used for the separation. The injector port temperature was 280 °C and the detector temperature was 280 °C. The initial temperature of the column was 40 °C which was gradually increased to 300 °C. The linear velocity of the helium carrier gas was 1 mL/min at a split ratio of 100:1; EI was used as the ion source, and the ion source temperature was 280 °C. The sector mass analyzer was set to scan m/z from 1.5 to 1890 amu, with a scan time of 1 s. Diluted samples (1 mL/15 mL) were prepared using ethanol as the solvent, and 500 µL samples were injected for analysis. Quantitative analyses of each essential oil component (expressed as a percentage area) were performed by peak area normalization. The components were identified by the Kovats retention index obtained from the sample retention time compared to that of standard hydrocarbons. The Kovat index was calculated as described by Hubschmann using the following equation (19).

$$I = 100Z + 100\left[\frac{\log_{t'R(x)} - \log_{t'Rz}}{\log_{t'R(Z+1)} - \log_{t'Rz}}\right] \quad (1)$$

Where I is the Kovats index, t'R(x) stands for the retention time of compound X, t'Rz indicates the retention time of n-alkane with carbon number Z appearing before compound X, t'R(Z+1) is the retention time of n-alkane with carbon number Z+1 appearing after compound X, X is the selected compound, Z is an n-alkane with the carbon number Z appearing before X, Z+1 is an n-alkane with carbon number Z+1 appearing after X.

The individual peaks/constituents were identified by comparing the calculated Kovats index with those in the literature.

Experimental animals

SD rats were obtained from an animal testing laboratory at the National Agency for Drug and Food Control (Indonesia) and acclimatized before treatment. Eight-week-old male and female rats weighing 190-230 g were used in this study. Prior to treatment, rats were randomly selected and tagged for further identification. The rats were given standard food (*ad libitum*) and maintained at 22 ± 3 °C, with a 75% relative humidity and a 12/12-h dark/light cycle. Female rats fulfilled the non-pregnancy criteria and had never been pregnant or delivered. The Institutional Animal Ethics Committee approved all the experimental protocols through Ethic No. 032-2019 KEH TROP BRC.

Acute oral toxicity studies

To study the acute toxicity of CREO, rats were randomly divided into four groups (5 rats each). Subsequently, CREO (2000, 4000, 8000, and 16000 mg/kg) was administered orally as a single dose, and the dose volume was maintained at 10 mL/kg. The rats were observed for 14 days for signs of morbidity and mortality. The lethal dose 50 (LD_{50}) was calculated using Weil's equation as follows:

$$\text{Log LD50} = \log D + d\,(f + 1) \quad (2)$$

where D is the lowest dose, d is the log of dose multiples, and f is a factor of 0.5.

Sub-acute oral toxicity studies

Thirty male and thirty female rats were used in this study. Each category (male or female) was randomly divided into six groups, including the control group, three treatment doses, one satellite for the control, and one satellite for the dose. Subsequently, the rats were orally administered with CREO at 50, 100, and 200 mg/kg body weight or distilled water (control) by oral gavage for 28 days. The dose volume was maintained at 10 mL/kg body weight; each dose was administered at 24-h intervals. The rats in the control and treatment groups were sacrificed on day 29. In contrast, the satellite groups were observed continuously without treatment for the next 14 days during the recovery period and then sacrificed.

Clinical signs and mortality

Clinical signs and mortality were observed daily during treatment, especially at 4 h, 24 h, and 28 days after sample administration. The satellite groups were observed for 14 days after administration of the last sample.

Body weight and food consumption

Daily food consumption and body weight of the rats were measured weekly after the inception of treatment and at the termination of the treatment schedule. These parameters were continually observed in the satellite groups for two weeks after the last treatment.

Hematological and biochemical parameters

The rats were euthanized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Blood samples were collected *via* cardiac puncture. Hematological tests included white blood cell (WBC), red blood cell (RBC), and platelet counts; hematocrit; RBC volume; differential WBC count; hemoglobin concentration; mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); and mean corpuscular hemoglobin concentration (MCHC). Biochemical parameters included alanine transaminase (ALT) and aspartate transaminase (AST), which were used to evaluate liver function, and creatinine and urea, which were used to evaluate kidney function.

Organ index and macroscopic and histopathological examinations

The major visceral organs, mainly the liver, spleen, heart, lungs, and kidneys, were inspected for gross morphological abnormalities. The organ indices (organ weight/body weight) were calculated. Organs were washed in saline solution, fixed in 4% paraformaldehyde solution, and embedded in paraffin for ease of slicing. Organ sections were stained according to the standard hematoxylin-eosin protocol and observed under a light microscope.

Statistical analysis

Data are presented as mean ± SEM. Analysis of variance and statistical data processing were performed using the one-way ANOVA followed by Tukey's post-hoc test. P-values ≤ 0.05 were considered statistically significant. All data analyses were performed using SPSS software.

RESULTS

Profile and phytochemical content of CREO

The *C. aeruginosa* rhizomes used in this study were fresh rhizomes with 85.95% moisture content. Qualitative phytochemical testing revealed the presence of alkaloids, saponins, and terpenoids in the *C. aeruginosa* rhizomes. Furthermore, GC/MS analysis of the CREO obtained after steam distillation of the *C. aeruginosa* rhizomes showed that the primary components were eucalyptol, epicurzerenone, and camphor, along with several other minor components (Table 1).

Acute oral toxicity of CREO

The results of the CREO acute toxicity tests in SD rats are presented in Table 2. In SD rats, administration of 2000 mg/kg CREO resulted in no symptoms of intoxication, and all animals survived the 14-day study period. In contrast, CREO administration at doses of 4000, 8000, and 16000 mg/kg resulted in 1, 3, and 4 mortalities, respectively, during the study period. According to the acute toxicity data collected using Weil's method, the LD_{50} of CREO was 5662 mg/kg; therefore, CREO was categorized as practically nontoxic.

Sub-acute oral toxicity of CREO

Clinical signs and mortality

The clinical signs and mortality observed during the sub-acute toxicity evaluation are shown in Table 3. No toxicity symptoms or mortalities associated with CREO administration were observed in rats, and the rats behaved normally and stayed active during treatment.

Table 1. *Curcuma aeruginosa* rhizome's essential oil (CREO) content based on GC/MS analysis.

Compound	Formula	Class	KIST	KICAL	RT (min)	Area (%)	Reference for KIST
α-Pinene	C10H16	Monoterpene	937	945	7.123	1.57	(20)
Camphene	C10H16	Monoterpene	953	964	7.647	3.49	(20)
β-Pinene	C10H16	Monoterpene	979	990	8.438	4.37	(20)
D-Limonene	C10H16	Monoterpene	1029	1039	9.858	1.98	(20)
Eucalyptol	C10H18O	Monoterpenoid	1033	1044	10.003	26.42	(20)
2-Nonanol	C9H20O	Alcohol	1092	1099	11.786	1.31	(20)
Camphor	C10H16O	Monoterpenoid	1144	1163	13.709	27.13	(20)
Isoborneol	C10H18O	Monoterpenoid	1164	1178	14.206	6.12	(20)
Endo-borneol	C10H18O	Monoterpenoid	1168	1185	14.441	1.43	(20)
L-4-Terpineol	C10H18O	Monoterpenoid	1182	1191	14.638	2.12	(22)
L-α-Terpineol	C10H18O	Monoterpenoid	1192	1203	15.027	3.18	(23)
β-Elemene	C15H24	Sesquiterpene	1387	1402	20.926	2.12	(24)
Curzerene	C15H20O	Sesquiterpenoid	1470	1508	23.823	1.05	(25)
Epicurzerenone	C15H18O2	Sesquiterpenoid	1623	1615	26.601	12.11	(26)
4-Pentyl-1-(4-propylcyclohexyl)-1-cyclohexene	C20H36	Hydrocarbon	-	1762	30.158	1,68	

KICAL, Calculated Kovats index; KIST, standard Kovats index; RT, retention time.

Table 2. Effect of *Curcuma aeruginosa* rhizome's essential oil (CREO) on the survival of rats during acute toxicity test.

Treatment (mg/kg)	Dead/total (n)	Dead (%)	Symptoms	LD_{50} (mg/kg)
2000	0/5	0	None	5662*
4000	1/5	20	Hypoactivity, asthenia	
8000	3/5	60	Hypoactivity, asthenia	
16000	4/5	80	Hypoactivity, asthenia	

*Practically nontoxic according to Weil's method.

Table 3. Effect of CREO on the survival of rats during sub-acute toxicity test.

Groups	Number of rats	Toxic symptoms	Dead	Number of rats with toxic symptoms
Male				
Control	5	None	0	0
CREO 50 mg/kg	5	None	0	0
CREO 100 mg/kg	5	None	0	0
CREO 200 mg/kg	5	None	0	0
Satellite group of control	5	None	0	0
Satellite group of CREO 200 mg/kg	5	None	0	0
Female				
Control	5	None	0	0
CREO 50 mg/kg	5	None	0	0
CREO 100 mg/kg	5	None	0	0
CREO 200 mg/kg	5	None	0	0
Satellite group of control	5	None	0	0
Satellite group of CREO 200 mg/kg	5	None	0	0

CREO, *Curcuma aeruginosa* rhizome's essential oil.

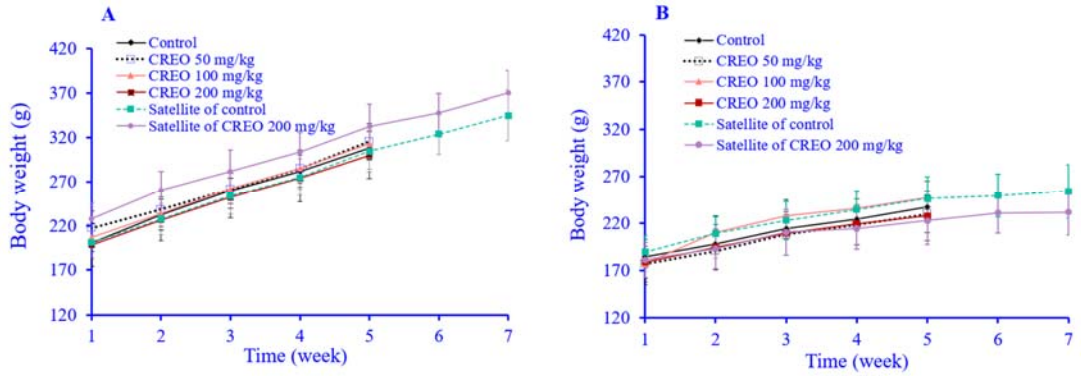

Fig. 1. Body weight increment of (A) male and (B) female rats during treatment. No significant differences in body weight were observed among the groups in the same week. CREO, *Curcuma aeruginosa* rhizome's essential oil.

Body weight and food consumption of rats

After 28 days, the body weights of the male and female CREO-treated rats did not change significantly compared to those of the control group. The weight of the rats increased weekly during the therapy and reversibility monitoring periods (Fig. 1). Male rats gained 4.5-15.65% of their body weight per week, whereas female rats gained 0.43-18.79% per week. Considering food consumption, CREO treatment in male rats tended to suppress appetite, without affecting weight. In contrast, female rats treated with low to medium doses of CREO showed an increase in appetite. This suggests that CREO may stimulate appetite in female rats (S1). However, this increase in appetite did not change the body weight of the female rats, indicating that the additional food intake was not converted into body weight gain.

Hematologic profile

Table 4 summarizes the hematological parameters and Table 5 shows the leukocyte profiles for all treatments. The results showed that the values of all hematological parameters, except the MCH of male rats and the RBC count of female rats, were at the expected intervals and did not significantly differ from those of the control group. A slight decrease was observed in the average MCH value of male rats in the group that received 200 mg/kg of CREO, and a subtle increase in the RBC count was observed in female rats in the satellite groups administered with CREO. However, these values remained within the expected intervals. No significant differences were observed in any of the leukocyte parameters, including lymphocyte, monocyte, and granulocyte counts.

Table 4. Effect of CREO on hematological parameters of rats during sub-acute toxicity test. Data are represented as mean ± SEM. [a, b, c]$P < 0.05$ indicate significant differences in the same column. The numbers under each hematological parameter represent the standard intervals for each parameter (27).

Groups	WBC (10^3/µL) 2.9-15.3	RBC (106/µL) 5.3-10	Hb (g/dL) 14-18	Hct (%) 36-46	Platelet (10^3/µL) 100-1.610	MCV (fL) 53-68.8	MCH (pg) 16-23.1	MCHC (g/dL) 30-34.1
Male								
Control	9.94 ± 2.33[a]	8.26 ± 0.19a	15.18 ± 0.26[a]	45.12 ± 0.82[a]	1128.60 ± 175.90[a]	54.74 ± 1.31[a]	18.34 ± 0.27[b]	33.58 ± 0.35[a]
CREO 50 mg/kg	7.18 ± 2.19[a]	7.62 ± 1.12a	13.96 ± 2.27[a]	41.96 ± 6.60[a]	1006.20 ± 483.71[a]	55.08 ± 1.19[a]	18.24 ± 0.61[ab]	33.20 ± 0.44[a]
CREO 100 mg/kg	7.20 ± 1.53[a]	8.08 ± 0.16a	15.16 ± 0.30[a]	45.22 ± 0.89[a]	1194.20 ± 134.63[a]	56.02 ± 1.51[a]	18.70 ± 0.26[b]	33.46 ± 0.62[a]
CREO 200 mg/kg	7.50 ± 2.90[a]	7.13 ± 1.63a	12.56 ± 3.15[a]	38.22 ± 9.01[a]	596.00 ± 465.60[a]	53.60 ± 0.86[a]	17.48 ± 0.49[a]	32.70 ± 0.78[a]
Satellite of control	7.08 ± 0.90[a]	8.29 ± 0.57a	15.28 ± 1.24[a]	45.96 ± 3.62[a]	970.00 ± 267.21[a]	55.48 ± 1.68[a]	18.38 ± 0.45[b]	33.18 ± 0.43[a]
Satellite of CREO 200 mg/kg	7.44 ± 1.89[a]	8.24 ± 0.38a	15.40 ± 0.64[a]	46.00 ± 1.99[a]	1123.40 ± 103.56[a]	55.90 ± 0.73[a]	18.66 ± 0.15[b]	33.44 ± 0.39[a]
Female								
Control	8.25 ± 1.30[a]	7.14 ± 7.59[a]	14.12 ± 0.22[a]	41.90 ± 0.82[ab]	984.00 ± 634.19[a]	58.77 ± 0.59[a]	19.75 ± 0.10[a]	33.65 ± 0.24[a]
CREO 50 mg/kg	5.19 ± 80.67[a]	6.94 ± 50.54[a]	16.20 ± 3.99[a]	40.40 ± 2.39[a]	942.60 ± 471.47[a]	58.34 ± 1.40[a]	23.80 ± 8.06[a]	40.74 ± 13.14[a]
CREO 100 mg/kg	8.45 ± 1.76[a]	7.50 ± 33.40[ab]	14.20 ± 0.45[a]	42.68 ± 1.47[ab]	1325.33 ± 99.98[a]	57.05 ± 1.46[a]	18.95 ± 0.60[a]	33.30 ± 0.36[a]
CREO 200 mg/kg	7.98 ± 1.12[a]	7.35 ± 20.42[ab]	14.23 ± 0.31[a]	42.67 ± 0.93[ab]	1349.33 ± 124.74[a]	58.18 ± 1.10[a]	19.43 ± 0.27[a]	33.52 ± 0.38[a]
Satellite of control	5.74 ± 1.24[a]	7.55 ± 29.23[ab]	14.62 ± 0.65[a]	43.56 ± 1.61[b]	1279.00 ± 104.98[a]	57.76 ± 0.83[a]	19.30 ± 0.31[a]	33.52 ± 0.33[a]
Satellite of CREO 200 mg/kg	6.88 ± 2.30[a]	7.79 ± 38.91[b]	14.72 ± 0.70[a]	43.50 ± 1.80[b]	1116.40 ± 560.30[a]	55.90 ± 1.28[a]	18.84 ± 0.55[a]	33.80 ± 0.38[a]

CREO, Curcuma aeruginosa rhizome's essential oil. WBC, White blood cell; RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Table 5. Leukocyte profiles during sub-acute toxicity test. Data were represented as mean ± SEM. [a, b, c]$P < 0.05$ indicate significant differences in the same column. The numbers under each leukocyte differential represent the standard intervals for each parameter (27).

Groups	Lymphocyte (%) 63.7 - 90.1	Monocyte (%) 1.5 - 4.5	Granulocyte (%) 7.3 - 30.1
Male			
Control	74.26 ± 7.51a	2.66 ± 0.53a	23.08 ± 7.60a
CREO 50 mg/kg	76.52 ± 3.48a	2.20 ± 0.34a	21.28 ± 3.29a
CREO 100 mg/kg	71.82 ± 5.16a	2.64 ± 0.39a	25.54 ± 4.84a
CREO 200 mg/kg	77.08 ± 2.52a	2.26 ± 0.45a	20.66 ± 2.60a
Satellite of control	67.82 ± 9.76a	2.90 ± 0.95a	29.28 ± 8.84a
Satellite of CREO 200 mg/kg	66.92 ± 4.44a	2.86 ± 0.53a	30.22 ± 4.11a
Female			
Control	72.65 ± 7.61ab	2.70 ± 0.60a	24.65 ± 7.79ab
CREO 50 mg/kg	78.54 ± 1.61b	2.18 ± 0.31a	19.28 ± 1.79a
CREO 100 mg/kg	71.62 ± 4.64ab	2.63 ± 0.35a	25.75 ± 4.36ab
CREO 200 mg/kg	73.00 ± 10.24ab	2.63 ± 0.99a	24.37 ± 9.37ab
Satellite of control	72.06 ± 4.88ab	2.48 ± 0.31a	25.46 ± 4.59ab
Satellite of CREO 200 mg/kg	65.00 ± 3.11a	3.00 ± 0.66a	32.00 ± 2.80b

CREO, *Curcuma aeruginosa* rhizome's essential oil.

Table 6. Value of the AST, ALT, urea, and creatinine of rats during sub-acute toxicity study (27,44,45). Data are represented as mean ± SEM. [a, b, c]$P < 0.05$ indicate significant differences in the same column. Numbers under each biochemical parameter represent the standard intervals for each parameter.

Groups	AST (U/L) 80-250	ALT (U/L) 1-223	Urea (mg/dL) 12.33-77.6	Creatinine (mg/dL) 0.2-1.2
Male				
Control	237.874 ± 21.45[a]	88.50 ± 10.97[ab]	47.03 ± 5.66[a]	0.86 ± 0.12[a]
CREO 50 mg/kg	165.60 ± 23.97[a]	84.40 ± 5.84[ab]	60.92 ± 5.34[b]	0.90 ± 0.05[a]
CREO 100 mg/kg	155.82 ± 11.89[a]	80.72 ± 14.73[ab]	50.64 ± 4.6[ab]	0.90 ± 0.09[a]
CREO 200 mg/kg	214.93 ± 125.13[a]	126.50 ± 77.59[b]	38.56 ± 9.83[a]	0.80 ± 0.03[a]
Satellite of control	145.32 ± 15.37[a]	61.17 ± 10.18[a]	47.21 ± 4.91[a]	0.87 ± 0.03[a]
Satellite of CREO 200 mg/kg	161.70 ± 30.59[a]	69.22 ± 7.12[ab]	47.97 ± 5.69[a]	0.89 ± 0.03[a]
Female				
Control	186.51 ± 35.36[d]	72.73 ± 10.10[b]	63.05 ± 4.88[b]	0.98 ± 0.03[a]
CREO 50 mg/kg	160.49 ± 15.71[cd]	61.11 ± 9.31[ab]	59.13 ± 4.67[b]	1.08 ± 0.04[b]
CREO 100 mg/kg	148.790 ± 13.76[bc]	56.73 ± 8.69[a]	60.27 ± 6.26[b]	1.01 ± 0.04[ab]
CREO 200 mg/kg	150.73 ± 10.82[c]	57.63 ± 4.54[ab]	57.27 ± 3.73[b]	0.94 ± 0.02[a]
Satellite of control	117.52 ± 8.39[ab]	56.20 ±4.46[a]	45.15 ± 2.63[a]	0.98 ± 0.04[a]
Satellite of CREO 200 mg/kg	112.72 ± 9.78[a]	59.21 ± 9.94[ab]	45.16 ± 7.12[a]	0.99 ± 0.07[a]

CREO, *Curcuma aeruginosa* rhizome's essential oil; AST, aspartate transaminase; ALT, alanine transaminase.

Blood biochemical parameters

Biochemical parameters, including AST, ALT, urea, and creatinine levels, are presented in Table 6. The results showed that the urea levels in male rats and AST, ALT, urea, and creatinine levels in female rats were significantly different from those in the control group, but remained within regular intervals. In male rats, AST, ALT, and creatinine levels were within the expected intervals and were not significantly different from those in the control group.

Organ index and macropathological and histopathological examinations

The effects of CREO on the organ index and histopathological sections of various organs are shown in Table 7, Figs. 2 and 3, and the macropathological appearance is provided Fig. S1 (Available at

https://github.com/IFajarwati/CA/blob/main/Supplementary%20file.docx). We did not observe any organ index increases in any of the groups compared to the control group. However, we found a significant decrease in organ index in some groups. However, they did not show any macropathological or histopathological changes. Histopathological changes were observed in the livers of male rats, particularly in those that received CREO at 200 mg/kg. Furthermore, their sinusoidal spaces were expanded because of edema and capillary congestion. The hepatocytes were larger than usual and had empty vacuoles in their cytoplasm, pushing the nucleus to the periphery and causing congestion in large blood vessels. A few mononuclear inflammatory cells were also observed in the perivasculature, indicating inflammation.

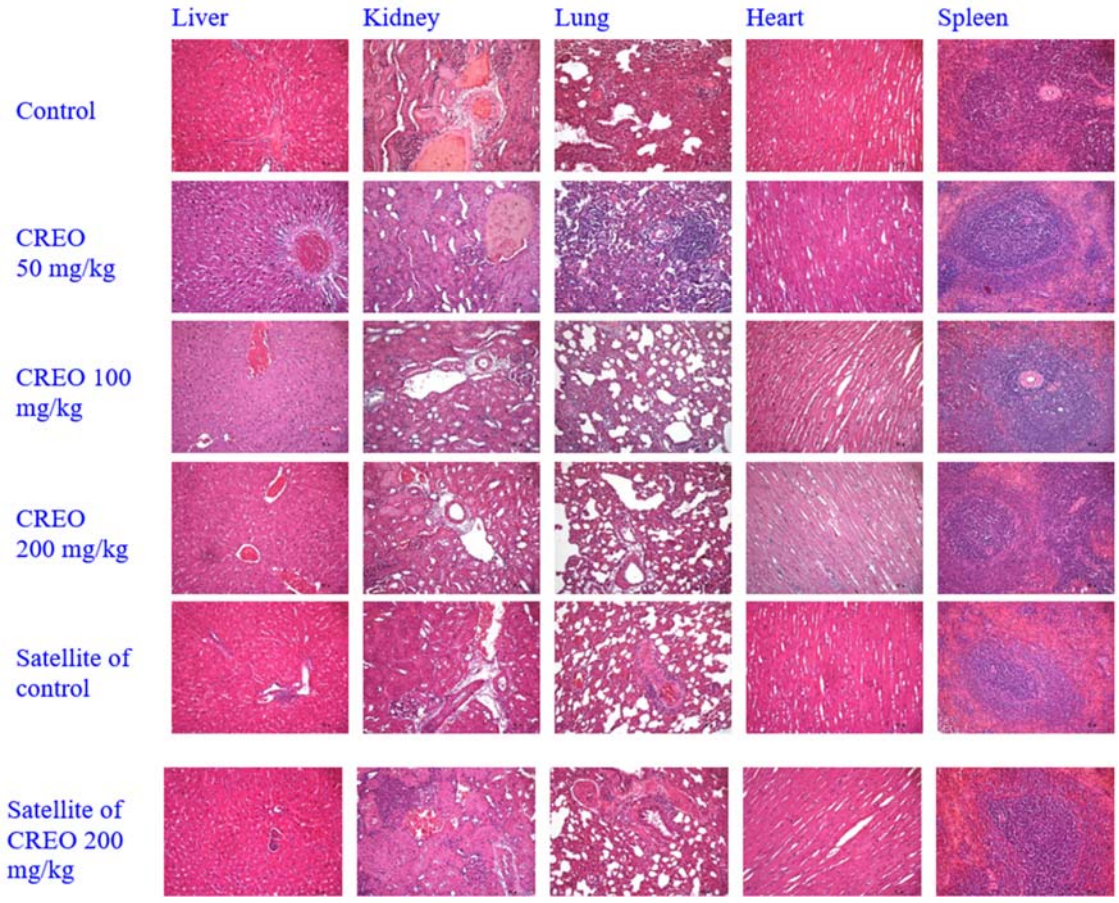

Fig. 2. Representative photomicrographs of the liver, kidney, lung, heart, and spleen sections stained with hematoxylin and eosin in the male rat group (magnification = 100×).

Table 7. Organ index of female and male rats treated with CREO. Data are represented as mean ± SEM. [a, b]$P < 0.05$ indicate significant differences in the same column.

Groups	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)		Heart (%)
				Left	Right	
Male						
Control	3.77 ± 0.50[a]	0.38 ± 0.07[b]	0.36 ± 0.04[b]	0.24 ± 0.03[a]	0.66 ± 0.10[b]	0.35 ± 0.04[a]
CREO 50 mg/kg	3.42 ± 0.21[a]	0.35 ± 0.04[ab]	0.35 ± 0.04[ab]	0.24 ± 0.05[a]	0.46 ± 0.04[a]	0.36 ± 0.06[a]
CREO 100 mg/kg	3.59 ± 0.27[a]	0.34 ± 0.03[ab]	0.32 ± 0.03[ab]	0.21 ± 0.02[a]	0.51 ± 0.09[a]	0.28 ± 0.01[a]
CREO 200 mg/kg	3.50 ± 0.18[a]	0.36 ± 0.02[ab]	0.35 ± 0.03[ab]	0.22 ± 0.01[a]	0.48 ± 0.07[a]	0.30 ± 0.02[a]
Satellite of control	3.38 ± 0.14[a]	0.31 ± 0.02[ab]	0.30 ± 0.03[ab]	0.20 ± 0.02[a]	0.43 ± 0.04[a]	0.30 ± 0.02[a]
Satellite of CREO 200 mg/kg	3.44 ± 0.16[a]	0.30 ± 0.01[a]	0.29 ± 0.03[a]	0.22 ± 0.03[a]	0.40 ± 0.02[a]	0.29 ± 0.04[a]
Female						
Control	4.17 ± 0.71[b]	0.40 ± 0.10[b]	0.39 ± 0.11[b]	0.28 ± 0.03[a]	0.55 ± 0.03[a]	0.50 ± 0.21[b]
CREO 50 mg/kg	3.60 ± 0.19[ab]	0.32 ± 0.02[ab]	0.33 ± 0.04[ab]	0.28 ± 0.03[a]	0.59 ± 0.04[a]	0.32 ± 0.02[a]
CREO 100 mg/kg	3.50 ± 0.43[ab]	0.30 ± 0.03[a]	0.30 ± 0.03[a]	0.26 ± 0.05[a]	0.52 ± 0.10[a]	0.32 ± 0.03[a]
CREO 200 mg/kg	3.60 ± 0.40[ab]	0.32 ± 0.04[ab]	0.32 ± 0.04[ab]	0.29 ± 0.03[a]	0.60 ± 0.06[a]	0.35 ± 0.02[a]
Satellite of control	3.53 ± 0.20[ab]	0.29 ± 0.02[a]	0.29 ± 0.01[a]	0.25 ± 0.03[a]	0.56 ± 0.07[a]	0.31 ± 0.01[a]
Satellite of CREO 200 mg/kg	3.41 ± 0.25[a]	0.30 ± 0.01[a]	0.29 ± 0.01[a]	0.24 ± 0.01[a]	0.53 ± 0.02[a]	0.33 ± 0.03[a]

CREO, *Curcuma aeruginosa* rhizome's essential oil.

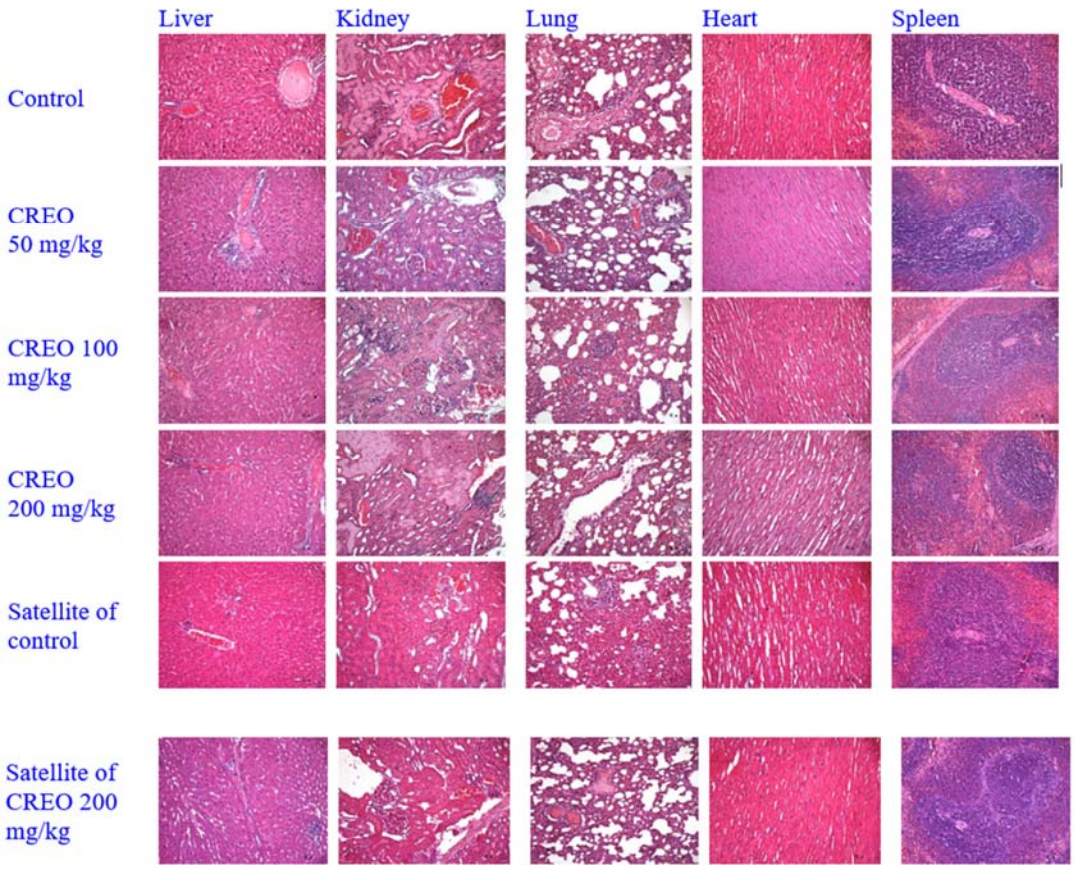

Fig. 3. Representative photomicrographs of the liver, kidney, lung, heart, and spleen sections stained with hematoxylin and eosin in the female rat group (magnification = 100×).

DISCUSSION

The present study evaluated the acute and sub-acute toxicity of CREO and quantified the primary bioactive metabolites by GC/MS. The acute toxicity evaluation did not show any abnormal activity or death after the administration of a single oral dose of 2000 mg/kg CREO. Some abnormal behavioral activities, such as hypoactivity and asthenia, were observed at higher doses of CREO (4,000, 8,000, and 16,000 mg/kg), and mortality gradually increased with increasing doses. Although mortalities were observed at very high doses, CREO can still be categorized as practically nontoxic because its LD_{50} is more than 5000 mg/kg. This evaluation provides preliminary evidence regarding the safety of CREO.

Prior to pharmacological validation and the development of a phytomedicine for any medicinal plant, determination of sub-acute toxicity is often required. This is part of the regulatory guidelines for evaluating the safety of pharmaceuticals, chemicals, pesticides, and other substances by various agencies, such as the FDA in the United States or the European Medicines Agency in the European Union (28). Therefore, demonstrating the safety of phytochemicals for human consumption is necessary (29). Sub-acute toxicity evaluation also reveals whether repeated daily doses lead to accumulation in the body, potentially reaching toxic levels and gradually affecting tissues and organs (30). In this study, sub-acute toxicity evaluation allowed us to assess the effect of CREO on hematological and biochemical parameters and organ structure. We observed neither mortality nor abnormal behavioral activity during the sub-acute toxicity evaluation. We observed a slight decrease in food consumption in female rats that received low-to-medium CREO doses. However, in the body weight assessment, all animals gained weight normally, and no significant changes in body weight were observed in any group.

Hematologic analysis is essential for identifying blood disorders and assisting in the diagnosis of various organ-specific and systemic diseases (31). It also evaluates alterations associated with ingested toxic substances (32). We found no significant changes in the WBC count, RBC count, Hb, Hct, platelet count, MCV, or MCHC in male groups. No significant differences were found in leukocyte profiles, including lymphocyte, monocyte, and granulocyte counts. Concomitantly, a significant reduction in MCH was observed in male rats treated with 200 mg/kg CREO, potentially indicating anemia (33). A lack of nutrients such as iron, folate, and vitamin B12 can also lead to anemia (34). A recent study reported that a decline in both MCH and MCV is a biomarker for anemia, ultimately leading to a decrease in MCHC (33). We did not observe any changes in the MCV and MCHC, excluding the potential diagnosis of anemia. During the reversibility observation period in the male satellite group treated with 200 mg/kg CREO, the MCH value was in the same range as that of the control. Although the male MCH values appeared to be statistically lower, these levels normalized rapidly and reached the biological range. Furthermore, MCV, MCHC, and Hb were in the normal range, excluding the potential diagnosis of anemia.

The liver and kidneys are frequently subjected to stress caused by various toxic xenobiotic compounds (32). Functional assessments of these two organs are critical for evaluating the safety of existing and new drugs or bioactive compounds (35). Multiple blood biomarkers are required to assess the health status of the liver and kidneys. Notably, AST and ALT are common biomarkers of liver function (16,36), and urea and creatinine are common biomarkers of kidney function. Despite their limited sensitivity and specificity, AST and ALT levels remain the gold standard indicators for evaluating liver function (32). High levels of these enzymes indicate hepatotoxicity, whereas decreased levels indicate enzyme inhibition (29,37). Given the data provided in Table 6, administration of CREO at 100 and 200 mg/kg significantly decreased AST in defined female groups in comparison with the control group. This reduction was statistically significant and more prominent in female rats. However, the enzyme levels remained within the normal physiological range. In the ALT assessment, male rats receiving the highest CREO dose (200 mg/kg) had slightly elevated ALT levels above the normal range. However, they were still not

significantly different from the control group. In addition, ALT levels were lower in the satellite group than in the CREO 200 mg/kg male group. This finding indicates that after stopping CREO administration, the liver could recover, as indicated by the decreased ALT levels in the satellite group treated with CREO. These results suggest that CREO administration at the tested doses does not harm the liver. However, further evaluation through organ index, macropathological, and histopathological assessments is necessary to confirm these results.

Changes in urea levels represent the primary indicator of acute renal injury, whereas creatinine is the primary indicator of acute renal insufficiency and only increases significantly after a substantial loss of renal function (38). Our results showed that these markers were within the normal ranges. However, a slight but non-significant increase in urea levels was observed among male rats and creatinine levels among female rats, particularly in the 50 mg/kg CREO treatment group. Changes in metabolite levels were independent of dose, suggesting that the tested CREO doses were safe for kidney function.

Organ index is another crucial indicator of toxicity that requires an integrated interpretation of macropathological and histopathological results. An increase in the index indicates hypertrophy, whereas a decrease indicates necrosis. We found that none of the groups showed a significant increase in the index for any organ. However, we observed significant decreases in the index for the hearts of female rats and spleens of both sexes, without any macropathological or histopathological changes. This indicates that these changes were likely irrelevant and did not affect organ integrity. No abnormalities were observed in renal index or histopathological changes.

Research on the toxicity of CREO is limited. However, one study suggests that the oil may not be entirely safe due to its potentially toxic effects. These concerns are based on the finding that CREO leads to increased PARP-1 expression in the liver, indicating necrosis (39). This raises questions about the overall safety profile of the oil.

However, in this study, CREO exhibited no significant toxic effects under the evaluated conditions. This could be associated with the U-shaped dose-response relationships, known as classical hormesis. Hormesis describes how a substance can have beneficial effects at low doses but may become harmful at higher doses (40). This dual response has been observed for many biological, toxicological, and pharmacological agents (41). However, further research is required to confirm this effect, including testing CREO at higher doses or over longer periods in sub-chronic and chronic studies.

The discrepancy between our findings and those of other studies could also be due to factors, such as the oil dose or concentrations used, species of animals tested, or variations in extraction methods, including distillation duration, temperature, and geographical origin of the extract, which can affect the chemical composition and safety profile of the oil (42,43).

CONCLUSION

The study found that CREO is safe for use in controlled doses. Its main bioactive components are eucalyptol, epicurzerenone, and camphor. No mortality or abnormal biological parameters were significantly observed in the acute or sub-acute toxicity evaluations. Furthermore, CREO did not significantly affect the body weight, relative organ weight, blood biochemistry, complete hematology, or differential leukocyte levels. No specific damage was observed in the liver, kidneys, spleen, heart, or lungs.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

I. Batubara contributed to the conceptualization, methodology, validation, resources, data curation, writing, review, and editing of the article, visualization, and project administration. I. Fajarwati contributed to the validation, formal analysis, investigation, data curation, writing, review, and editing of the article, and visualization. Y.W. Sari contributed

to the conceptualization, resources, writing, review, and editing of the article, project administration, and funding acquisition. G.J. Guillemin contributed to the writing, review, and editing of the article and visualization. I. Maulidya contributed to the methodology, formal analysis, data curation, and visualization. D. Iryawati contributed to the investigation, writing, review, and editing of the article. W.T. Wahyuni contributed to conceptualization, methodology, validation, formal analysis, investigation, writing - original draft, writing, review, and editing of the article, and visualization. I. Batubara and W.T. Wahyuni contributed equally to this work.

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