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Original Article

Essential oil of *Ageratum conyzoides* (L.) L.: Acute and subchronic toxicity studies

[Aceite esencial de *Ageratum conyzoides* (L.) L.: Estudios de toxicidad aguda y subcrónica]

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Abstract

Context: The essential oil of the *Ageratum conyzoides* (100 mg/kg BW) previously demonstrated anti-neuropathic pain activity with various mechanisms of action. However, the safety data of this essential oil is unknown.

Aims: To evaluate the acute and subchronic toxicity profile of the *A. conyzoides* essential oil.

Methods: The acute toxicity study was conducted in both genders of Swiss Webster mice and monitored for 14 days of general behavior, LD50, organ-body weight ratio, and histopathology with sequence dose 1200, 1600, 2000, 2400 mg/kg BW. To determine the subchronic toxicity, 100 mg/kg BW was administered daily for 28 days in both genders of rats. The parameters that were determined include general behavior, body weight, organ-body weight ratio, biochemical and hematological.

Results: The acute oral toxicity test unveiled the respective LD50 values of 1247.88 mg/kg BW and 1674.57 mg/kg in female and male mice, respectively. The subchronic toxicity test did not result in any death in all groups. The only toxic sign observed was retching and disappearing in about five minutes. However, most of the abnormalities were found in male rats. These were lower kidney weight, increased creatinine, lower hemoglobin, erythrocyte, and hematocrit.

Conclusions: The essential oil of *A. conyzoides* at 100 mg/kg is considered unsafe due to concerns about its effects on the kidney and several hematologic parameters, and warrants attention in its future use.

Keywords: acute toxicity test; essential oils; subchronic toxicity test.

Resumen

Contexto: El aceite esencial de *Ageratum conyzoides* (100 mg/kg de peso corporal) ha demostrado anteriormente su actividad contra el dolor neuropático con diversos mecanismos de acción. Sin embargo, se desconocen los datos de seguridad de este aceite esencial.

Objetivos: Evaluar el perfil de toxicidad aguda y subcrónica del aceite esencial de *A. conyzoides*.

Métodos: El estudio de toxicidad aguda se realizó en ratones Webster suizos de ambos sexos y se controló durante 14 días el comportamiento general, la DL_{50}, la relación órgano-peso corporal y la histopatología con dosis secuenciales de 1200, 1600, 2000 y 2400 mg/kg de peso corporal. Para determinar la toxicidad subcrónica, se administraron diariamente 100 mg/kg de peso corporal durante 28 días a ratas de ambos sexos. Los parámetros que se determinaron incluyen comportamiento general, peso corporal, relación órgano-peso corporal, bioquímica y hematología.

Resultados: La prueba de toxicidad oral aguda reveló valores respectivos de DL_{50} de 1247,88 mg/kg de peso corporal y 1674,57 mg/kg en ratones hembra y macho, respectivamente. La prueba de toxicidad subcrónica no produjo ninguna muerte en todos los grupos. El único signo tóxico observado fue el vómito que desapareció en unos cinco minutos. Sin embargo, la mayoría de las anomalías se observaron en ratas macho. Éstas fueron un menor peso de los riñones, un aumento de la creatinina y un descenso de la hemoglobina, los eritrocitos y el hematocrito.

Conclusiones: El aceite esencial de *A. conyzoides* a 100 mg/kg se considera inseguro debido a la preocupación por sus efectos sobre el riñón y varios parámetros hematológicos, y merece atención en su uso futuro.

Palabras Clave: aceites esenciales; ensayo de toxicidad aguda; ensayo de toxicidad subcrónica.

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INTRODUCTION

Neuropathic pain has contributed to the global burden of illness, and the prevalence of this condition globally reached up to 10% (Colloca et al., 2017). New drug discovery and development for this condition are very important due to the side effects and inconvenience that might accompany the currently available drugs (Bonicalzi and Canavero, 2006; Brooks and Kessler, 2017; Cohen et al., 2015).

Ageratum conyzoides (L.) L. herbal medicine family *Compositae* is widely used in many countries with a long history of practicing herbal medicines (Okunade, 2002). Many pharmacological activities have been shown with the use of this plant, including antimicrobial, anti-inflammatory, analgesic, antioxidant, anticancer, antiprotozoal, antidiabetic, spasmolytic, and allelopathy (Vigil de Mello et al., 2016; Yadav et al., 2019). In our previous study, the essential oil of this plant at the dose of 100 mg/kg, which contains 60 compounds, showed anti-neuropathic pain activity via mu-opioid receptor activation, ATP-sensitive potassium channel activation, and GABA level elevation (Sukmawan et al., 2020; 2023). The anti-neuropathic pain activity of this component was comparable to pregabalin, which was used as the first-line treatment for neuropathic pain (Brooks and Kessler, 2017). The three major compounds in the A. conyzoides essential oil were precocene II, longifolene, and caryophyllene (Sukmawan et al., 2020; 2023). Precocene II has been demonstrated to affect reproductive activity, antijuvenile hormone development, digestive system, and fatty acid metabolism in insects (Chen et al., 2005; Fodor et al., 1982; Garcerá et al., 1989; Pratt and Bowers, 1977; Tarrant et al., 1982; Triseleva, 2007). Meanwhile, longifolene, and caryophyllene were shown to have pharmacological activities, which include lipase inhibitor, anti-mycobacterial, phytotoxicity, and anti-neuropathic pain activity (Gordien et al., 2009; Jassbi et al., 2010; Pedersen and Miller, 1999; Sukmawan et al, 2023; Wang et al., 2017). The results of the anti-neuropathic pain study of *A. conyzoides* essential oil were promising for the next stage of drug development. However, the safety profile of the essential oil is unknown. Thus, this study aimed to evaluate the acute and subchronic toxicity of *A. conyzoides* essential oil.

MATERIAL AND METHODS

Chemicals, reagents and kits

Spectrophotometer (Agilent Technology Cary 60), Hematology Analyzer (SFRI H-18), Microscope Binocular (XSZ 107 BN), vacuum tube lithium heparin (PT Berliantama Diagnostika, Indonesia), microtome (HP 35, Thermo Scientific, Singapore), staining jar (Hellendhal, Duran, Germany), syringe 1 mL (Terumo, Indonesia), glucose assay kit (Sigma Adrich Pte. Ltd., Singapore), creatinine assay kit (Sigma Aldrich, Pte. Ltd, Singapore), AST (Elabscience, PT Indogen Intertama, Indonesia), ALT (Elabscience, PT Indogen Intertama, Indonesia), and CK-MB (Sigma Aldrich, Pte, Ltd, Singapore), NaCl 0.9% (Otsu, Indonesia), alcohol 96% (PT. Brataco, Indonesia), xylol (Sigma Aldrich, Pte. Ltd, Singapore), hematoxylin (Sigma Aldrich, Pte. Ltd, Singapore), eosin (Sigma Aldrich, Pte. Ltd, Singapore), paraffin wax, pastilles (Sigma Aldrich, Pte. Ltd, Singapore), distilled water (PT Brataco, Indonesia), hexane (PT Brataco, Indonesia).

Essential oil preparation

The essential oil used is the same essential oil that was used in the previous study (Sukmawan et al., 2020). *A. conyzoides* leaves were obtained from Singaparna, Tasikmalaya, West Java, Indonesia, and identified at the Herbarium of Padjadjaran University, Bandung, West Java, Indonesia.

The fresh leaves of about 10 kg were washed using running water, dried and ground to powder, and placed in a distillation flask. Water was then added in a ratio of 5:1 to the ground leaves. Upon heating, the essential oils would float above the hydrosol. The distilled oil from the distillate was then extracted using hexane (2: 1), which was repeated 3 times and followed by the collection of the supernatant. The supernatant was then evaporated with a rotary evaporator at 35°C (Pratama et al., 2017; Sukmawan et al., 2020). The amount of harvested essential oil after evaporation or yields was as much as 0.31% (Sukmawan et al., 2020). Additionally, the ten largest chemical composition of this essential oil is precocene II; 7 azabicyclo[4.2.2]de-ca-2,4,7,9-tetraene,5,8-dimethoxy; longifolene; chromone; (3R,3aR,7R,8aS)-3,8,8-Trimethyl-6-methyleneoctahy-dro-1H-3a, 7 methanoazulene; caryophyllene; (3R, 3aR,7R,8aS)-3,8,8-trimethyl-6-methyleneoctahydro-1H-3a, 7-methanoazulene; isogermacrene D; (1S,2E,6E,10R)- 3,7,11,11-tetramethylbicyclo[8.1.0]undeca-2,6-diene; benzenepropanoyl bromide (Sukmawan et al., 2020).

Animals

All procedure was conducted according to the National Institutes of Health guide for the care and use of laboratory animals and approved by the Health Research Ethics Committee of Bakti Tunas Husada Health Science College with approval No. 05/kepk-bth/04/2020. Animals were provided by Bakti Tunas

Husada Health Science College, Tasikmalaya, West Java, Indonesia. Male and female Swiss Webster mice weighing 20-30 g and aged 6-8 weeks were used in the acute toxicity study, while male and female Wistar rats weighing 120-200 g and aged 6-8 weeks were used for the subchronic toxicity study. All animals were kept under standard laboratory conditions (25°C of room temperature, 30-70% of room humidity, 12 h light/12 h dark cycle) and free access to food and water. All the animals were sacrificed by cervical dislocation on days 14th and 28th for acute and subchronic toxicity studies, respectively.

Acute toxicity study

The acute toxicity test refers to the procedure established by Indonesia National Regulatory Authority (NRA) on guidelines for *in vivo* non-clinical toxicity tests (Indonesia Food and Drugs Administration, 2014). Male and female mice were randomly assigned into five groups (5 animals per group), namely control (vehicle), Test 1 (1200 mg/kg of essentials oil), Test 2 (1600 mg/kg of essentials oil), Test 3 (2000 mg/kg of essentials oil) and Test 4 (2400 mg/kg of essentials oil). The time rime of all the groups that received the treatment was 9 am to 1 pm. There were ten mice in each group, five from each sex. The animals were observed for signs of toxicity every 30 min for 4 h on the first day and continued once daily for 14 days. The safety information obtained from acute toxicity tests included general monitoring such as skin, eyes, mucous membrane, respiratory system, seizure, salivation, diarrhea, sedation, coma, and death. The organ-body weight ratio, $LD_{50,}$ and organ histopathology were also monitored. The collected organs for histopathology included kidneys, liver, and heart. The histopathology procedure was performed and described below.

Surgery and organ harvesting were done using surgical tools and performed with care to determine the organ damages. The organs were then followed by physiological NaCl 0.9% washed. After completion, the organs were fixed in a bottle containing formalin 10%. The organ was then washed with distilled water and immersed in a solution of FAA (formaldehyde: acetic acid: alcohol) for 24 h, then followed by dehydration, which was graded with ethanol 70, 80, 90, 95%, and absolute alcohol, respectively for 1 h. After dehydration, then continued 1-hour safe purification by immersing organs in an ethanol solution of xylol for 20 min. After the organs looked clear, followed by immersing organs in liquid paraffin with a melting point of 48-52°C, 52-54°C, and 54-56°C for 1-2 h (Cardiff et al., 2014).

After being infiltrated, the organs were implanted in the mold containing liquid paraffin until hardened. Then the organs in the paraffin were cut with a 4-5 µm thickness. The next step was deparaffinization by immersing deep tissue xylol for 10 min followed by a coloring process, then successively put into alcohol 96, 90, 80, 70, 60, 50, 40, and 30% each for 5 min, then to distilled water for 5 min and washed with running water for approximately 2 min. The sample was entered into hematoxylin Ehrlich 4 min later, and washed with running water for 10 min, and entered into distilled water and ethanol 50, 60, 70, 80, 90, and 96%. Then immersed in the eosin yellow 0.5% (in 70% alcohol) for 1.5 min and dipped in alcohol 70, 80, 90, 96%, and absolute ethanol for 10 min. The next step is preparation drain and put in xylol for 15 min (Cardiff et al., 2014).

The last preparation step was to give labels and descriptions and then sample observation under a microscope. Changes in kidneys, liver, and heart were observed under the microscope at magnifications 40-fold, 40-fold, and 100-fold, respectively.

28-day repeated dose toxicity study

The 28-day repeated dose toxicity test refers to the procedure established by the Indonesia National Regulatory Authority (NRA) on guidelines for *in vivo* non-clinical toxicity tests (Indonesia Food and Drugs Administration, 2014). Rats of both sexes were divided into two groups (5 animals per group), namely the control (vehicle) and test group (100 mg/kg of essential oil). Each group received the respective treatment once daily for 28 days (Indonesia Food and Drugs Administration, 2014). The time range of all the groups that received the treatment was 9 am to 1 pm. The rats were observed daily for toxicity signs and once a week for body weight. On day 28, the biochemical assays for glucose, creatinine, AST, ALT, and CK-MB through animal blood serum according to kit instructions for each biochemical determination were then determined using a spectrophotometer (Agilent Technology Cary 60). In addition, the biochemical assays for hemoglobin, leucocyte, erythrocyte, thrombocyte, and hematocrit were made in animal blood serum and then determined using a hematology analyzer (SFRI H18).

Statistical analysis

Statistical analysis was performed with ANOVA, followed by *post-hoc* analysis (Least Significant Difference) using SPSS version 16.00. If the data was not homogenous, non-parametric methods were used (Kruskal Wallis followed by Mann-Whitney). A difference was significant if the p-value was less than 0.05.

RESULTS

General profile of acute toxicity test of the essential oil of *A. conyzoides*

The LD_{50} of the essential oil of *A. conyzoides* was found to be lower in females than in males, as much as 1247.88 mg/kg BW and 1674.57 mg/kg BW, respectively. The death in male mice of groups 1 and 2 was observed on the third day and the second day in mice of group 3. All mice in group 4 died within 24 h after drug administration. In female mice, death in groups 1 and 2 occurred on the second day (3 death in group 2), and all mice in groups 3 and 4 died on the second day. Moreover, we also found an imbalance, sedation, inactivity, and, in rare conditions, jumping and convulsion. A summary of these results is in Table 1.

The effects of the essential oil of *A. conyzoides* on organ weight and organ histopathology

In Table 2, the essential oil component of the *A. conyzoides* affected the weight of the liver at a dose of 2400 mg/kg BW in male mice, as shown by a significant increase compared to the control ($p<0.05$). Yet, no significant increases were observed in the weight of other organs ($p>0.05$). In the female group, changes were observed in the kidney, liver, and lung weights, but not the heart, after all doses of the essential oil. Meanwhile, in Fig. 1, the results of the quantitative analysis per 1000 cells showed necrotic cells of the kidney, liver, and heart that increased with increasing doses. The respective number of necrotic cells per 1000 cells in the kidney, liver, and hearts after control and test group 1 through 4 were 54, 76, 81, 98, and 102; 67, 105, 101, 121, and 132; and 43, 83, 76, 98, and 104, respectively. In addition, hydropic and lipid degenerations were observed in a dose-dependent manner. The results of the qualitative analysis showed local bleeding and infiltration of the inflammatory cells in the kidney, liver, and heart after all tested doses. However, the 2400 mg/kg BW dose induced extensive bleeding (liver and the kidney) and infiltration of the inflammatory cells (liver).

General profile of 28-day repeated dose toxicity test of the essential oil of *A. conyzoides*

The subchronic toxicity test of the essential oil for 28 days showed no deaths in the control and test group (100 mg/kg). The rats of both sexes demonstrated the toxic sign of retching soon after the essential oil administration but disappeared 5 min later. On the third day, this sign disappeared after the oral administration of the essential oil. The summary of the subchronic toxicity profile is shown in Table 3.

The effects of the essential oil of *A.conyzoides* on the body weight and organ weight

The administration of the essential oil for 28 days in the sub-chronic toxicity did not induce any significant difference in body weight as compared to the control group ($p>0.05$) in both male and female rats (Table 4). Moreover, the organ weight of the female rats was not a significant difference compared to the control group. However, in male rats, the weight ratio of the heart and kidney was significantly decreased in the test group compared to the control (Table 5).

Table 1. General profile of acute toxicity of the essential oil of *A.conyzoides* leaves.

Sex	Route	Group (treatment)	Death (%)	Toxic signs
Male	Oral	Control	0	None
		Test 1 (EO 1200 mg/kg)	20	Retching, inactivity, diarrhea (2 mice)
		Test 2 (EO 1600 mg/kg)	20	Retching, imbalance, sedation, inactivity, slow breath
		Test 3 (EO 2000 mg/kg)	80	Retching, imbalance, sedation, inactivity, slow breath
		Test 4 (EO 2400 mg/kg)	100	Retching, imbalance, sedation, inactivity, slow breath, convulsion (1 mouse)
Female	Oral	Control	0	None
		Test 1 (EO 1200 mg/kg)	40	Retching, inactivity
		Test 2 (EO 1600 mg/kg)	80	Retching, inactivity, imbalance, sedation
		Test 3 (EO 2000 mg/kg)	100	Retching, imbalance, sedation, inactivity, slow breath
		Test 4 (EO 2400 mg/kg)	100	Retching, imbalance, sedation, inactivity, jumping (1 mouse)

All the behavior observation was compared to the control groups. EO: Essential oil.

Table 2. Effects of the essential oil of *A.conyzoides* on organ weight in acute toxicity test**.**

Sex	Group (Treatment)	Organ-to-body weight ratio (%)			
		Heart	Kidney	Liver	Lung
Male	Control	0.50 ± 0.05	1.32 ± 0.19	6.20 ± 0.69	0.69 ± 0.11
	Test 1 (EO 1200 mg/kg)	0.47 ± 0.05	1.59 ± 0.12	5.63 ± 0.91	0.88 ± 0.06
	Test 2 (EO 1600 mg/kg)	0.48 ± 0.04	1.46 ± 0.23	7.36 ± 1.62	0.86 ± 0.24
	Test 3 (EO 2000 mg/kg)	0.63 ± 0.18	1.74 ± 0.21	6.90 ± 0.69	0.97 ± 0.21
	Test 4 (EO 2400 mg/kg)	0.60 ± 0.11	1.85 ± 0.43	7.56 ± 1.31*	1.09 ± 0.32
Female	Control	0.67 ± 0.24	1.24 ± 0.14	5.03 ± 1.16	0.73 ± 0.20
	Test 1 (EO 1200 mg/kg)	0.79 ± 0.25	1.80 ± 0.17*	7.04 ± 1.62*	1.12 ± 0.26*
	Test 2 (EO 1600 mg/kg)	0.61 ± 0.13	1.99 ± 0.39*	8.67 ± 0.94*	1.50 ± 0.27*
	Test 3 (EO 2000 mg/kg)	0.66 ± 0.08	2.19 ± 0.17*	8.73 ± 1.73*	1.49 ± 0.22*
	Test 4 (EO 2400 mg/kg)	0.63 ± 0.08	1.80 ± 0.43*	7.14 ± 1.58*	1.26 ± 0.22*

Data represent mean ± SD (n = 5). *p< 0.05 *vs.* control, one-way ANOVA followed by post-hoc test LSD. EO: Essential oil.

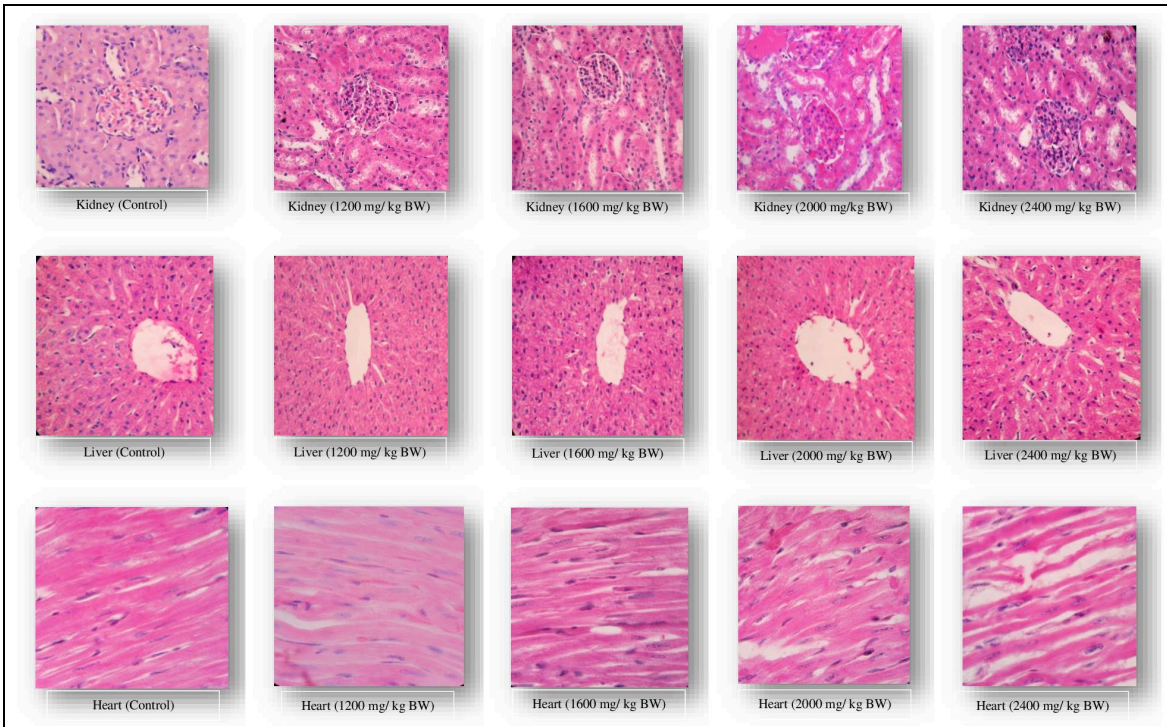

Figure 1. Organ histopathology in acute toxicity test.

The results of the quantitative analysis per 1000 cells showed necrotic cells of the kidney, liver, and heart, which were increased with increasing doses. In addition, hydropic and lipid degenerations were observed in a dose-dependent manner.

Effects of the essential oil of *A. conyzoides* on the biochemical and hematological parameters in a subchronic toxicity study

The administration of the essential oil for 28 days did not show differences in biochemical parameters in female rats. However, in male rats, there was a significant change in creatinine level ($p<0.05$) (Table 6). Meanwhile, the hematological parameters showed an increase in hemoglobin in female rats and a decrease in hemoglobin, erythrocyte, and hematocrit in male rats (Table 6). These findings might be related to the kidney damage found in male rats.

Table 3. General profile of subchronic toxicity test.

Sex	Group (treatment)	Death	Toxic signs
Male	Control	None	None
	Test (EO 100 mg/kg)	None	Retching disappeared in 5 min, absent on the third day
Female	Control	None	None
	Test (EO 100 mg/kg)	None	Retching disappeared in 5 min, absent on the third day

All the behavior was compared to the control group. EO: Essential oil.

Table 4. The effects of essential oil on body weight in a 28-day repeated dose study.

Sex	Groups (Treatment)	Days				
		0	7	14	21	28
Male	Control	177.72 ± 12.62	181.63 ± 16.02	186.86 ± 19.01	163.28 ± 18.69	158.02 ± 25.66
	Test (EO 100 mg/kg)	158.88 ± 16.69	165.51 ± 25.72	173.46 ± 29.62	163.42 ± 28.54	161.05 ± 24.81
Female	Control	111.08 ± 13.04	114.96 ± 13.99	111.32 ± 11.22	126.74 ± 11.52	117.62 ± 11.51
	Test (EO 100 mg/kg)	124.84 ± 35.47	125.10 ± 35.10	127.26 ± 36.51	113.92 ± 31.39	130.58 ± 32.64

Data represent mean ± SD (n = 5). EO: Essential oil.

Table 5. The effects of the essential oil on the organ weight in a 28-day repeated dose study.

Sex	Organ	Control (%)	Test (EO 100 mg/kg) (%)
Female	Liver	4.61 ± 0.74	4.10 ± 0.33
	Heart	0.40 ± 0.05	0.42 ± 0.09
	Lungs	1.18 ± 0.28	1.20 ± 0.25
	Right kidney	0.51 ± 0.12	0.44 ± 0.05
	Left kidney	0.49 ± 0.13	0.45 ± 0.02
	Spleen	0.75 ± 0.23	0.68 ± 0.11
Male	Liver	4.18 ± 0.42	4.02 ± 0.55
	Heart	0.38 ± 0.03	0.32 ± 0.04*
	Lungs	1.07 ± 0.20	0.87 ± 0.34
	Right kidney	0.47 ± 0.04	0.37 ± 0.02*
	Left kidney	0.43 ± 0.03	0.37 ± 0.03*
	Spleen	0.75 ± 0.20	0.60 ± 0.17

Data represent mean ± SD (n = 5). *p<0.05 vs control, one-way ANOVA followed by post-hoc test LSD. EO: Essential oil.

DISCUSSION

The acute and 28-day repeated dose toxicity tests are intended to determine the safety of the essential oil of *A. conyzoides*. The LD_{50} values of acute toxicity study in both groups are classified as mildly toxic based on the criteria of the Indonesia Food and Drugs Administration (2014). The female mice were found to be more susceptible to the toxic effects of the essential oil. Anatomical, pharmacokinetics, and pharmacodynamics have been proposed to contribute to the susceptibility of female subjects (Mennecozzi et al., 2015; Miller, 2001; Torres-Rojas and Jones, 2018). Anatomical aspects include body weight, height, surface area, total body water, extracellular water, and intracellular water, which are lower in females than males (Soldin and Mattison, 2009). From the pharmacokinetics viewpoint, females are more likely to experience overdose than males due to the smaller volume of distribution, larger free drug fraction, and slower clearance. Meanwhile, from the pharmacodynamics standpoint, females are more sensitive due to the alteration in receptor number, receptor binding, and signal transduction pathway (Miller, 2001; Soldin and Mattison, 2009). Furthermore, the higher androgen level in males tends to speed cardiac repolarization

Table 6. Biochemical and hematological parameters in rats 28-day repeated dose toxicity study of the *A. conyzoides* essential oil.

Sex	Parameters	Control	Test (EO 100 mg/kg)
Female	Glucose (mg/dL)	105.55 ± 20.46	107.65 ± 5.03
	Creatinine (mg/dL)	0.79 ± 0.13	0.87 ± 0.47
	AST(U/L)	241.01 ± 167.48	180.33 ± 61.49
	ALT (U/L)	178.10 ± 28.97	221.63 ± 121.53
	CK-MB (U/L)	1396.43 ± 555.77	614.47 ± 131.83
	Hemoglobin (g/dL)	10.72 ± 2.34	12.98 ± 3.33*
	Leucocyte (cells/µL)	8060 ± 2966.00	5600 ± 1757.00
	Thrombocyte (cells/µL)	89000 ± 36789.00	96000 ± 35496.00
	Erythrocyte (cells/µL)	4.85 ± 0.82	5.71 ± 1.38
	Hematocrit (%)	25.78 ± 6.19	30.14 ± 7.62
Male	Glucose (mg/dL)	104.17 ± 40.29	125.49 ± 15.93
	Creatinine (mg/dL)	0.61 ± 0.11	0.88 ± 0.15*
	AST(U/L)	141.50 ± 38.94	121.46 ± 70.82
	ALT (U/L)	69.52 ± 64.26	87.10 ± 59.62
	CK-MB (U/L)	1740.25 ± 720.35	2071.75 ± 109.42
	Hemoglobin (g/dL)	17.64 ± 1.21	12.68 ± 1.90*
	Leucocyte (cells/µL)	4133 ± 351.00	4340 ± 1543.00
	Thrombocyte (cells/µL)	221600 ± 35203.00	170000 ± 124904.00
	Erythrocyte (cells/µL)	7.75 ± 0.53	5.31 ± 1.25*
	Hematocrit (%)	39.70 ± 4.21	28.24 ± 5.22*

Data represent mean ± SD (n = 5). *p<0.05 vs control, one-way ANOVA followed by post-hoc test LSD. EO: Essential oil.

and shorten the QT interval, which contributes to the protection from toxic events (Miller, 2001). Many therapeutic agents cause QT interval-prolongation (Miller, 2001), and *A. conyzoides* crude extracts demonstrated changes in the electrocardiogram, heart impulse, and coronary blood vessel resistance (Garcia and Carvalho, 1999; Soldin and Mattison, 2009). D-limonene, linalool, linalyl acetate, a-pinene, dibutyl phthalate, caryophyllene, geranyl acetate, α-terpineol, terpinene-4-ol, and α-santalol may cause sedation of the study animals (Bommareddy et al., 2019; Buchbauer et al., 1991; Faturi et al., 2010; Umezu et al., 2006; Zhong et al., 2019). A previous study showed that *A. conyzoides* essential oil contained a-pinene, caryophyllene, and limonene (Sukmawan et al., 2020). The sedative effect of essential oil may be related to the walking imbalance observed in the test. Essential oil such as thujone, 1,8-cineole, camphor, or pinocamphone, have been identified as convulsive agents (Bahr et al., 2019), but these compounds were not identified in the essential oil component of *A. conyzoides* (Sukmawan et al., 2020). However, among the 60 compounds contained in the essential oil, a yet unknown component might be responsible for the convulsions observed in the present toxicity study.

The findings of the increase in the liver organ weight and extensive bleeding in acute toxicity test on this organ might be associated with the content of precocene II in *Ageratum conyzoides* essential oil as the major compound (Dees et al., 1982; Duddy and Hsia, 1987; 1989; Hammond and Fry, 1992; 1994; 1995). The toxic effects of precocene II to the liver have been suggested through the depletion of glutathione and inhibition of cytochrome P-450, peaking in 24 h, then decreasing in 72 h (Hammond and Fry, 1992; 1994). In 24 h precocene II exposure to hepatocytes induced 65% death of the cells, followed by rapid loss of ATP in 72 h (Hammond and Fry, 1995). In addition, precocene II also increased superoxide levels in mitochondria leading to mitochondrial damage (Furukawa et al., 2015).

In the subchronic toxicity test, the only behavior toxic sign observed was retching. The retching sign may indicate an unpleasant feeling in the upper gastrointestinal tract (Yamamoto et al., 2017; Wang et al.,

2019). However, in organ weight ratio, biochemical parameter, and hematological parameter, we found a decrease in the kidney and heart organ, increased creatinine, and a decrease of hemoglobin, erythrocyte, and hematocrit in male rats. These findings might be related to α2u-globulin nephropathy (Bastaki et al., 2020; Swenberg, 1993), which was unique to male rats that are associated with exposure to an ever-increasing number of chemicals, but not considered predictive of risk to humans (Doi et al., 2007; Swenberg, 1993). The finding that showed a renal abnormality that occurred in male rats might be related to the altered renal clearance of the essential oil in males leading to renal accumulation (Soldin and Mattison, 2009). Caryophyllene and limonene contained in *Ageratum conyzoides* essential oil are indicated to induce α2u-globulin nephropathy (Bastaki et al., 2020; Swenberg, 1993). Moreover, decreased hematological parameters might be associated with the absence of erythropoietin due to kidney damage (Babitt and Lin, 2012; Mehdi and Toto, 2009).

CONCLUSION

The essential oil of *Ageratum conyzoides* at 100 mg/kg is considered unsafe due to concerns about its effects on the kidney and several hematologic parameters and warrants attention in its future use.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Adnyana IK	Anggadiredja K	Sukmawan YP
Concepts or ideas	x	x	x
Design	x	x	x
Definition of intellectual content	x	x	x
Literature search	x	x	x
Experimental studies	x	x	x
Data acquisition	x	x	x
Data analysis	x	x	x
Statistical analysis	x	x	x
Manuscript preparation	x	x	x
Manuscript editing	x	x	x
Manuscript review	x	x	x

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